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14. ABSTRACT

The presence of a fibrotic focus in breast tumors is associated with a 10-50-fold increase in tissue stiffness and correlates with distant metastasis and poor outcome. Recent studies indicate that increasing tissue rigidity promotes breast cancer progression, however the underlying molecular mechanism is largely unknown. Breast cancer stem cells have both long-term self-renewal capacity and the ability to initiate tumors. In this proposal, we hypothesize that tissue rigidity regulates breast cancer stem cell properties and function, therefore assisting breast tumor development and promoting chemoresistance.

In the past year, our major findings are the following.

1. We have established two 3D culture systems to mimic the physiological ranges of matrix rigidities from normal mammary gland to breast cancer.
2. We have tested whether increasing matrix stiffness promotes CSC properties.
3. We have established the mammary implantation models and determined the proper dose of LOX inhibitor BAPN to be used in vivo to effectively reduce matrix stiffness.
4. We have found that increasing matrix stiffness promotes Epithelial-MesenchymalTransition(EMT) in human breast cancer cells and this regulation depends on β1 integrin and the EMT-inducing transcription factor Twist1.
5.

Together, these results indicate that increasing matrix stiffness promotes EMT via an integrin and Twist1-dependent pathway to regulate breast cancer stem cell function, thus impacting breast cancer progression.

15. SUBJECT TERMS
Matrix stiffness, breast cancer stem cell, Epithelial-Mesenchymal Transition (EMT).

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INTRODUCTION

Breast tumors are frequently detected through physical palpation as a rigid mass residing within the soft normal mammary tissue. The presence of a fibrotic focus in breast tumors is associated with a 10-50-fold increase in tissue stiffness and correlates with distant metastasis and poor outcome. Recent studies indicate that increasing tissue rigidity promotes breast cancer progression, however the underlying molecular mechanism is largely unknown. Breast cancer stem cells have both long-term self-renewal capacity and the ability to initiate tumors. In this proposal, we hypothesize that tissue rigidity regulates breast cancer stem cell properties and function, therefore assisting breast tumor development and promoting chemoresistance. Therefore, the proposed research aims to determine the impact of matrix stiffness on breast cancer stem cell function and to understand the molecular mechanism underlying this regulation. Given the critical role of breast cancer stem cells in breast tumor progression and chemoresistance, our research could lead to novel therapeutics targeting the mechanotransduction pathway to eradicate breast cancer stem cells and overcome chemoresistance.

BODY

Task 1. Determine the impact of matrix stiffness on breast cancer stem cell function, Months 1-18:

1. Determine the role of matrix stiffness on regulating breast cancer stem cell properties in 3D mammary culture, Months 1-6.

a. Establish the 3D hydrogel culture systems for harvesting large numbers of cells for FACS analysis, Month 1-2 (Dr. Engler's group).

Dr. Engler's group has successfully developed the methods to produce two types of hydrogels on which to culture mammary epithelial cells, one with static properties as proposed (polyacrlyamide) and also one with dynamic properties that could remodel with time (hyaluronic acid); the dynamically stiffening material better mirrors the temporal nature of tumor stiffening. Both systems can fully mimic the physiological ranges of tissue rigidities from normal mammary gland (~150Pa) to human breast cancer (~5000Pa). In both systems, matrix stiffness can be accurately defined independently of biochemical factors, such as concentration of ECM proteins and growth factors.

The first system is the 3D PA-Matrigel overlay culture system, where matrix stiffness (or substrate elasticity) is defined by a polyacrylamide (PA) base with calibrated elastic moduli ranging between the ~150 Pascal (Pa) of normal mammary glands and the ~5700 Pa of breast tumor tissues (1, 3). The PA layer is cross-linked with type I collagen to allow cell attachment. Non-transformed human MCF10A and tumorigenic mouse Eph4Ras mammary epithelial cells were used in our studies. These cells were then cultured on top of this matrix and overlaid with 2.5% reconstituted Matrigel basement membrane (4). We found that this 3D PA-Matrigel culture system allows the formation of polarized mammary ductal acini in the compliant "soft" matrix, while rigid matrix stiffness induced an EMT-like phenotype including loss of epithelial polarity, and degradation of basement membrane, and loosening of cell-cell adhesion (Fig. 6D), consistent with previous publications (1).

One significant problem with model systems that rely on polyacrylamide and/or matrigel to recapitulate the mammary niche is that they present constant niche properties to mammary cells, which is not the case with cancer. Indeed previous landmark efforts exploring these properties (1) highlight the importance of intrinsic niche properties like stiffness where cells became more malignant in nature when the surrounding extracellular matrix (ECM) stiffness exceeded 500 Pascals (Pa; a unit of stiffness) or 3-fold stiffer than native mammary tissue. However, a critical issue with their system underscores cell sensitivity to stiffness: the niche is not stiff from the outset. In their system polyacrylamide hydrogels were used, and this synthetic ECM presents cells with constant properties. Mammary acini do not develop in a niche with tumor-like stiffness, e.g. 500–5000 Pa. Rather this stiffening occurs after tissue maturation and mammary acini formation. Thus in parallel with the grant activities, the Yang and Engler labs have also pursued creating dynamic hydrogels that stiffen on demand to pose the similar question as Paszek et al but in a more biomimetic niche: "Does the mature mammary acinar structure desensitize mammary epithelial cells to changes in matrix stiffness?"

To accomplish this, we substituted a previously developed hyaluronic acid (HA) hydrogel that was modified with a UV-sensitive methacrylate (Figure 1A) to permit "on demand" free radical polymerization ((2); Figure 1B). HA hydrogels were then functionalized with a thin layer of Matrigel to permit attachment, MCF10A mammary epithelial cells were then seeded onto the HA, and cells were were coated in a second layer of Matrigel to create a 3D niche (Figure 1C) similar to Paszek et al but using a different underlying substrate. When MCF10A cells were allowed to mature in HA hydrogels with a single round of crosslinking, cells on stiffer matrices (2000 Pa) underwent EMT whereas those on soft (100 Pa) did not (Figure 1D). Again, soft and stiff niche were created by partially or completely crosslinking the HA hydrogel (Figure 1E, F).

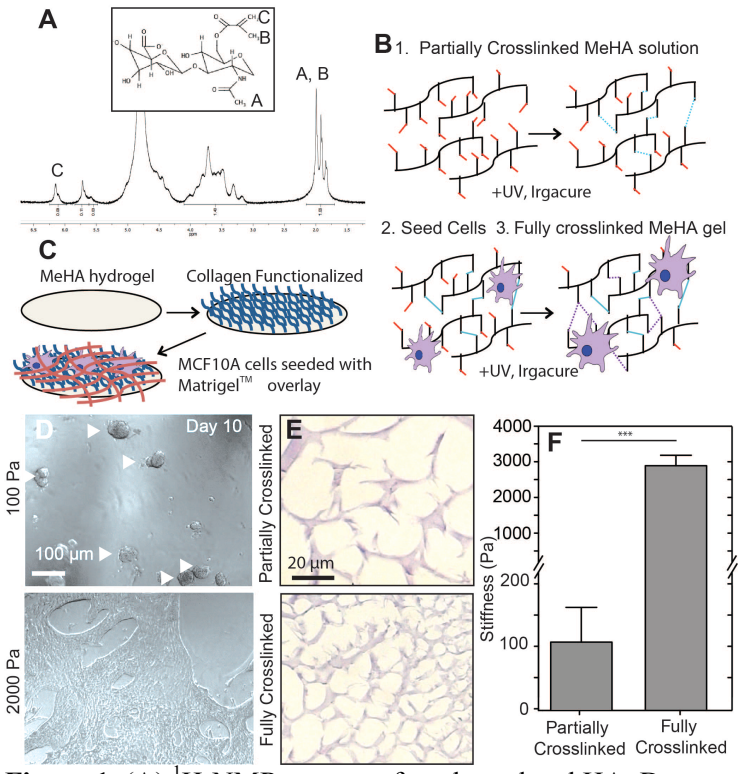

Figure 1. (A) ^{1}H-NMR spectra of methacrylated HA. Degree of substitution is determined from a ratio of peak C divided by peaks A or B. (B) Schematic of polymerization scheme. (C) Schematic of hydrogel functionalization and Matrigel overlay scheme. (D) Brightfield images of MCF10A cells cultured on HA hydrogels of the indicated stiffness. White arrowheads indicate mature acinar structures. (E) Histology of the HA hydrogels to indicate crosslinking. (F) HA hydrogel stiffness before and after complete crosslinking.

However, the system can be partially crosslinked at first, cells

seeded and allowed to mature, and then upon formation of acini, it can be crosslinked; this scheme (outlined in Figure 1B, C), better recapitulates the timing of stiffening *in vivo* than what Paszek et al previous performed. Thus we plated MCF10A cells on substrates pre-stiffening (static), post-stiffening (static), or a pre-stiffened hydrogel which we crosslinked after 10 days in culture (dynamic) to transition from soft to stiff (Figure 2A). Cells that were plated on soft substrates that remained soft throughout the culture time remained in acinar structures (Figure 2B, left). Cells on stiff substrates from the outset of the culture underwent EMT as in Paszek et al (Figure 2B, right). However the longer that MCF10A cells were cultured on soft substrates before stiffening, the more likely they were to resist responding to the stiffened substrate and undergo EMT once the HA hydrogel had been stiffened (Figure 2B, center). Figure 2C quantifies this where cells on hydrogels stiffened at day 10 remain more resistant to stiffness-induced EMT than those stiffened earlier.

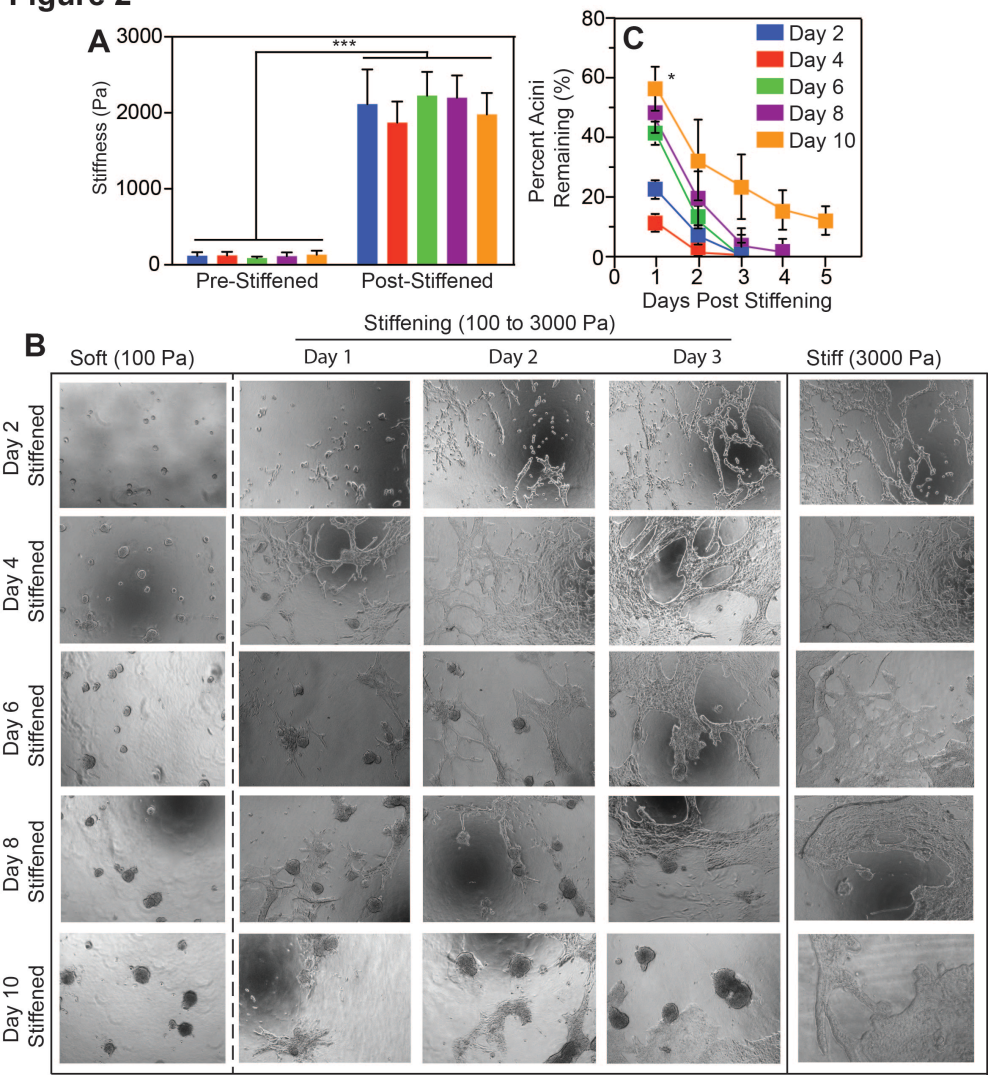

Figure 2. (A) Stiffness of HA hydrogels before and after a second free radical polymerization to stiffen the hydrogel. ***p<0.001. (B) Brightfield images of MCF10A cells in the indicated matrix conditions (columns) and time course when stiffened (rows). (C) Percentage of acini present versus day 0. *p<0.05 from ANOVA comparisons versus day stiffened.

b. Use Anchorage-independent mammosphere assay to determine the impact of matrix stiffness on breast CSC mammosphere forming ability, Months 2-6 (Dr. Yang's group).

To understand whether matrix stiffness regulates anchorage-independent mammosphere forming abilities of breast cells, we used both non-transformed human MCF10A and tumorigenic mouse Eph4Ras mammary epithelial cells in the 3D PA-Matrigel culture system. Upon 7 days of culture, we incubated the cultures first in PBS-EDTA solutions at 4C to dissolve the Matrigel and harvested through collagenase/dispase/tryspin digestion to obtain single cells in suspension. Then single cells were cultured in serum-free MEGM media (Lonza) in the ultra-low attachment plates at 150K cells/well and the number of mammospheres was quantified in triplicated wells. As a positive control, we used HMLE-Twist1 cells in the mammosphere formation assay given the known role of EMT in promoting mammosphere formation (5). Upon primary mammosphere formation, we then performed secondary mammosphere formation assay to further evaluate the stem cell properties of the cells. Interestingly, at Day 4 and Day 9 after seeding, we observed significantly more mammopheres formed by cells from the 5700Pa culture than from the 150Pa culture (Figure 3A). Upon quantification of multiple repeats, we found that cells from 5700Pa culture generated about 4-fold more mammospheres than cells from the 150Pa culture (Figure 3B). Together, these results suggest that increasing matrix stiffness significantly increases the mammosphere formation ability in breast cancer cells.

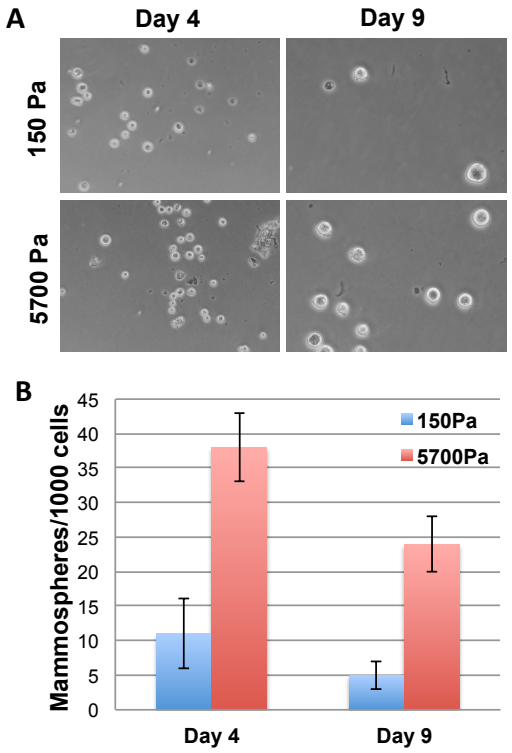

Figure 3. Increasing matrix stiffness promoted mammosphere formation. A) Human MCF10A cells were cultured in the PA-Matrigel 3D culture for 7 days and then collected into single cell suspension. Then single cells were cultured in serum-free MEGM media (Lonza) in the ultra-low attachment plates at 150K cells/well. B) The number of mammospheres was quantified in triplicated well at Day 4 and Day 9.

c. Use FACS analysis of ALDEFLUOR and CSC cell surface markers to test the impact of matrix stiffness on breast CSC properties, Months 5-8 (Dr. Yang's group).

In addition to using the mammosphere formation assay to directly analyze the functionality of breast cancer stem cells, a variety of surface markers and other molecular markers can be used to further evaluate the changes of breast cancer stem cell properties in response to tissue rigidities. Among them, CD44highCD24low has been the most widely used markers for human breast cancer stem cells. Therefore, we performed the 3D PA-Matrigel culture of human breast cells MCF10A described above and used FACS analyses to measure the percentage of CD44highCD24low populations under different matrix rigidities. Surprisingly, we found that MCF10A cells contained 33.1% of CD44highCD24low population when cultured in 2D regular tissue culture dishes. When they were cultured in the PA-Matrigel 3D culture at 150Pa rigidity, the CD44highCD24low population increased significantly to 64.2%. Similarly,

when the same cells were cultured in the PA-Matrigel 3D culture at 5700Pa rigidity, the CD44highCD24low population increased significantly to 66.7% (Table I). Together, these data show that the 3D Matrigel condition significantly shifts the cell population from CD24high to CD24low. It suggests that the shift towards CD44highCD24low is likely needed for cells to adapt to growth in the 3D suspension culture; however, CD44highCD24low alone is not sufficient to mark cells with cancer stem cell properties, as we originally proposed. Therefore, we are currently testing additional markers and ALDEFLUOR assays to determine whether additional markers would allow us to more specifically enrich cancer stem cell population.

conditions	$CD24^{high}$ $CD44^{low}$	$CD24^{high}$ $CD44^{high}$	$CD24^{low}$ $CD44^{low}$	$CD24^{low}$ $CD44^{high}$
MCF10A unstained	1.2%	0	98%	0.7%
MCF10A 2D	0	66.9%	0	33.1%
MCF10A 150 Pa	0.2%	35.3%	0.3%	64.2%
MCF10A 5700 Pa	0.1%	33.2%	0.1%	66.7%

Table I. The effect of the PA-Matrigel 3D culture condition on CD44highCD24low population. MCF10A cells were cultured in the PA-Matrigel 3D culture or in 2D plastic dishes for 7 days and then analyzed for CD44 and CD24 expression by FACS analysis. The MCF10A cells without staining with CD44 or CD24 antibodies were used negative control for gating. The percentages of cell populations with different CD44 and CD24 levels are listed.

Published literatures show that different cancer stem cells can be enriched with very diverse members of molecular markers and it is unclear whether any of these markers play a biological role in regulating cancer stem cell function. Therefore, we decided to focus more on using functional assays (including mammosphere formation and tumor initiation assay in vivo) to characterize cancer stem cells, instead of relying on molecular markers. Therefore, we put more efforts on Task 2 that was originally proposed for Year 2 and 3.

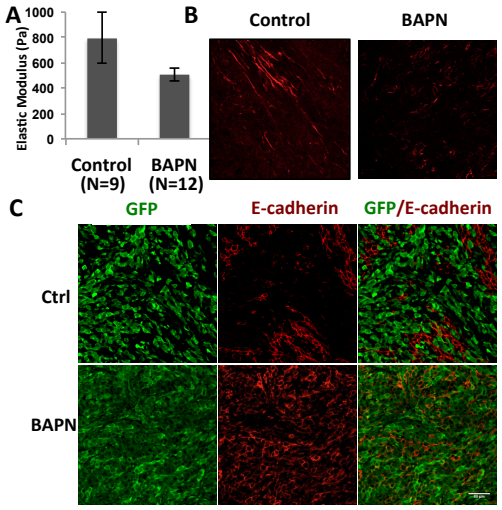

Figure 4. BAPN treatment reduces matrix stiffness, fibrillar collagen and EMT phenotypes in breast tumor xenografts. A) BAPN treatment reduced equilibrium modulus calculated between 5% and 15% strain in primary tumors. B) SHG imaging confirmed reduced straight fibrillar collagen in primary tumors upon BAPN treatment. C) BAPN treatment resulted in more epithelial tumors with higher E-cadherin expression. Tumor cells were labeled with GFP.

2. Determine whether rigid matrix stiffness promotes tumor initiation efficiency in vivo, Months 9-18.

a. Establish mammary implantation models and determine the proper dose of BAPN treatment on matrix stiffness in vivo, Months 9-11 (Dr. Yang's group).

Towards this goal, we have implanted EPH4Ras cells in the mammary fat pads and treated half of the mice daily with β-aminopropionitrile (BAPN), a non-reversible LOX inhibitor (6, 7), to inhibit collagen crosslinking. We tested various dose used and the delivery routes (orally through drinking water vs. intraperitoneal injection). In collaboration with Dr.

Robert Sah at UCSD Bioengineering, Dr. Albert Chen in his group has adapted the equipment and analysis software to measure and analyze the elastic modulus of tumor samples by unconfined compression testing (8). Our preliminary data showed that the equilibrium elastic modulus of the EPH4Ras tumor samples was in average 800Pa and that BAPN treatment reduced the elastic modulus of EPH4Ras tumor samples by 40% without obvious toxicity (Fig.4A). To further evaluate whether BAPN reduces fibrillar collagen in tumors, we used two-photon-excited Second-Harmonic Generation (SHG) microscopy to image and quantify fibrillar collagen curvature ratio (9) in unstained tumor sections and confirmed the effect of BAPN (Fig. 4B). Furthermore, BAPN treatment resulted in more epithelial tumors with higher E-cadherin expression (Fig. 4C). We found that intraperitoneal injection at a dose of 100 mg/kg in 100 µl PBS resulted in the best LOX inhibition with less side effects. Therefore, we have successfully established the condition for BAPN treatment in vivo.

Task 2. Determine the mechanotransduction pathways that regulate cancer stem cell in response to matrix stiffness, Months 19 – 36:

Although we initially planned to pursue this aim in the 2nd and 3rd year of the funding cycle, we have made very interesting observations on the role of matrix stiffness in regulating epithelial-mesenchymal transition (EMT) via activating the EMT-inducing transcription factor Twist1 soon after starting this project. Given that the EMT program has been tightly linked to giving rise to breast cancer stem cell properties (5, 10), we decided to pursue this mechanistic aim immediately ahead of schedule given the critical role of EMT in breast cancer progression.

1. Determine the role of known mechanosensing pathways in regulating cancer stem cell function, Months 19-30.

a. Test whether β1 integrin and its downstream kinases are requried for transmitting matrix stiffness to CSC regulation in the 3D PA-Matrigel assays, Months 19-23 (Dr. Engler and Dr. Yang's group).

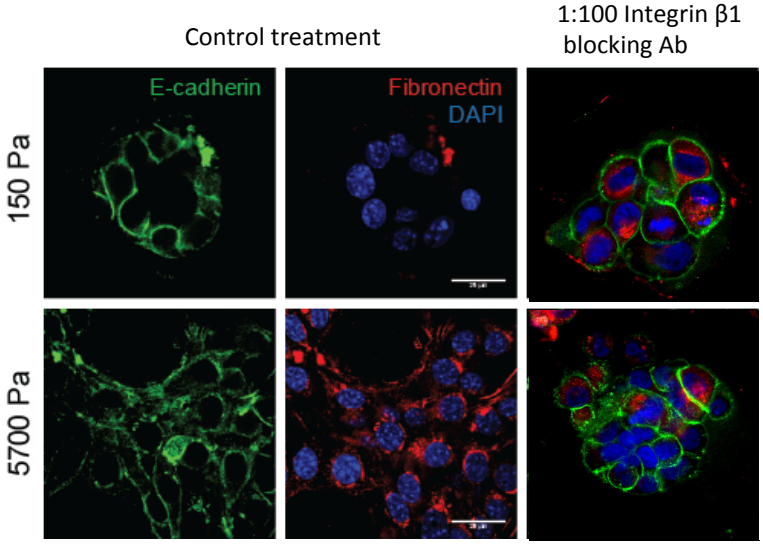

Figure 5. β1 integrin activation is required for induction of the EMT phenotype in response to the rigid matrix stiffness. A blocking antibody against β1 integrin or a control IgG was added in the 3D acini culture of MCF10A cells and stained for E-cadherin (green), fibronectin (red), and nuclei (blue).

Toward this goal, we first analyze whether matrix stiffness promotes EMT by examining two EMT markers E-cadherin and firbonectin. We found that this 3D PA-Matrigel culture system allows the formation of polarized mammary ductal acini in the compliant "soft" matrix, while rigid matrix stiffness induced an EMT phenotype including loss of epithelial polarity, weakening of epithelial marker E-cadherin, gain of mesenchymal marker fibronectin, and degradation of basement membrane. Mechanosensing responses to matrix

stiffness are in part mediated through clustering and activation of integrins (11). To begin to understand how mechanical forces regulate EMT, thus impacting breast cancer stem cell properties, we first tested the involvement of integrin signaling in response to increasing matrix stiffness. Singnificantly, a monoclonal antibody against b1 integrin(1, 7, 12) blocked the EMT phenotype in response to the rigid matrix stiffness, as observed by the maintenance of E-cahderin expression and the absence of fibronectin upregulation. Furthermore, it also rescued the ability of mammary epithelial cells to form spheroid ductal acini (Fig. 5). Together, these data suggest that induction of EMT is regulated by mechanical force in a β1 integrin-dependent manner.

2. Test the involvement of Epithelial-Mesenchymal Transition in regulating breast cancer stem cell in response to matrix stiffness, Months 22-36.

a. Use shRNA lentivirus to knock down individual genes in human breast cancer cell lines and test their effects on CSC properties in response to rigid matrix stiffness, Months 24-28 (Dr. Yang's group).

To understand whether EMT-inducing transcription factors plays functional roles in the mechanosensing response, we have tested whether knocking down individual EMT-inducing transcription factors blocks the EMT-like phenotype induced by rigid matrix stiffness. There are three major families of EMT-inducing transcription factors, Twist1/2, Snail1/2, and Zeb1/2. Based on the availability of shRNAs against these factors, we have tested shRNAs again Twist1 and Snail2 to date. We used two independent shRNAs to knock down endogenous Twist1 (Fig. 6A) or Snail2 expression in MCF10A and EPH4Ras cells and applied the resulting cells to the 3D mammary acini cultures with matrix stiffness arranging from 150 Pa to 5700 Pa. Significantly, in both cell types, knockdown of Twist1, but not Snail2, prevented the EMT-like invasive phenotype induced by the stiff matrix stiffness of 5700Pa; instead these mammary cells formed spheroid mammary ductal acini similar to that in the compliant matrix stiffness of 150Pa (Fig.6B-6D). Since high stiffness alone was not sufficient to induce a complete EMT, we further investigated whether Twist1 is also required for the induction of a full EMT by mechanical signals in concert with the EMT-inducing biochemical

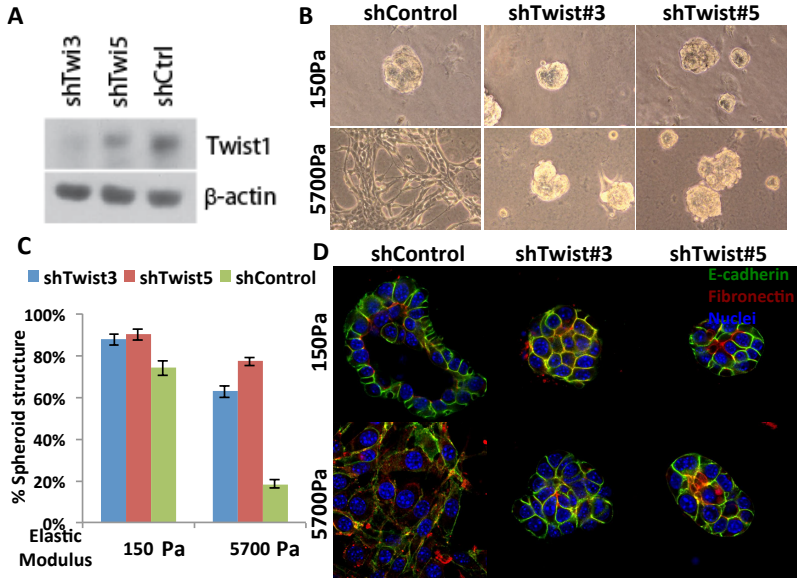

Figure 6. Twist1 is required for rigid matrix stiffness-induced EMT phenotype. A) Western blot analysis shows the knockdown level of Twist1 by two independent shRNAs in EPH4Ras cells. B-C) Knocking down Twist1 blocked EMT-like invasion and recued spheroids formation in rigid matrix in EPH4Ras cells. D) Immunostaining of EPH4Ras cells shows that knocking down Twist1 prevented loosing of adherent junctions as evident by E-cadherin (green) membrane localization.

signal TGF-beta(13). Indeed, knockdown of Twist1 also completely blocked induction of EMT by TGF-beta at high matrix stiffness and rescued acinar development (data not shown). Together, these results indicate that Twist1 is a key player in a cellular mechanosensing pathway and plays an essential role in mediating EMT in response to matrix stiffness.

KEY RESEARCH ACCOMPLISHMENTS

- We developed two hydrogel systems and determined their mechanic properties.
- We found that increasing tissue rigidities promoted breast cancer stem cell properties.
- We established the LOX inhibitor treatment conditions to effectively inhibit collagen crosslinking in mice.
- We uncovered that a mechanistic link between tissue rigidity and breast cancer stem cells was via the activation of the EMT program.
- We found that the EMT-inducing transcription factor Twist1 was essential for high tissue-rigidity-induced EMT.

REPORTABLE OUTCOMES

1. Presentations:

2013/11	The 6th International EMT meeting, Alicante, Spain
2014/01	IRCCS Istituto Clinico Humanitas, Milan, Italy
2014/01	The ISREC Symposium on "Metastatic colonization: micro-environments, mechanisms, and therapeutic targeting", Crans Montana, Switzerland
2014/02	Keystone Symposium on "Stem Cells and Cancer", Banff, Canada
2014/03	University of California, Irvine, CA

Manuscripts: Two review articles were published with partial support from this grant.
Paz H, Pathak N, and Yang J. (2013) Invading one step a time: the role of invadopodia in tumor metastasis. *Oncogene*. Advance online publication doi: 10.1038/onc.2013.393.

Tsai JH, and Yang J. (2013) Epithelial-Mesenchymal plasticity in carcinoma metastasis. *Genes & Development*. 27 (20): 2192-206.

2. A graduate student Spencer Wei who pioneered this project just defended his PhD thesis in March 2014.

CONCLUSION

Results from our first year of proposed research have identified a mechanotransduction pathway that transmits the mechanical cues from the breast tumor microenvironment to influence breast cancer stem cell properties via activation of Twist1 and the EMT program. Breast tumors are often detected through physical palpation due to their apparent "hardness" compared to their normal compliant tissues. The presence of a fibrotic focus in breast tumors is a prognostic marker of distant metastasis and correlates with poor survival. Besides the biochemical factors from tumor stroma, fibrotic tumor lesions are associated with a 20-50 fold increase in tissue rigidity. Combining cell and molecular biology techniques with new bioengineering research tools, we have begun to uncover a novel mechanotransduction pathway that link tissue rigidity to breast cancer stem cell function.

Not only does understanding the impact of tissue rigidity on breast cancer stem cells enhance our knowledge of the molecular regulation of cancer stem cell, it also has direct impact on breast tumor prognosis and cancer treatment. Since cancer stem cells are thought to be responsible for breast tumor initiation and progression, genes and pathways involved in mechanoregulation of cancer stem cells holds promise to be useful prognostic markers for breast cancer. Given the critical role of breast cancer stem cells in breast tumor progression and chemoresistance, our research could lead to novel therapeutics targeting the mechanotransduction pathway to eradicate breast cancer stem cells and overcome chemoresistance.

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REVIEW

Epithelial–mesenchymal plasticity in carcinoma metastasis

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Tumor metastasis is a multistep process by which tumor cells disseminate from their primary site and form secondary tumors at a distant site. Metastasis occurs through a series of steps: local invasion, intravasation, transport, extravasation, and colonization. A developmental program termed epithelial–mesenchymal transition (EMT) has been shown to play a critical role in promoting metastasis in epithelium-derived carcinoma. Recent experimental and clinical studies have improved our knowledge of this dynamic program and implicated EMT and its reverse program, mesenchymal–epithelial transition (MET), in the metastatic process. Here, we review the functional requirement of EMT and/or MET during the individual steps of tumor metastasis and discuss the potential of targeting this program when treating metastatic diseases.

Epithelial and mesenchymal cell types have long been recognized by their unique cell morphology and organization in tissues. Epithelial cells form polarized sheets or layers of cells that are connected laterally via several types of cellular junctions, including adherens junctions, desmosomes, and tight junctions. In addition, epithelial cells anchor themselves to the underlying basement membrane via hemidesmosomes to maintain apical–basal polarity. Both desmosomes and hemidesmosomes further connect with the epithelial-specific cytokeratin intermediate filaments. In contrast, mesenchymal cells embed themselves inside the extracellular matrix (ECM) and rarely establish tight contact with neighboring cells. During specific embryonic morphogenesis processes such as mesoderm formation and neural crest development, epithelial cells can exhibit enormous plasticity and transit into a mesenchymal state by activating the epithelial–mesenchymal transition (EMT) program. After EMT, these cells lose their epithelial junctions and switch to producing vimentin filaments. The functional hallmark of the EMT program is to allow stationary epithelial cells to gain the ability to migrate and invade during developmental morphogenesis (Boyer and Thiery 1993; Hay 1995).

Although epithelial cells convert into the mesenchymal state during developmental EMT, entering the EMT program is not necessarily an irreversible commitment, as evident during kidney tubule formation. These epithelial cells can activate a transitory EMT program and then undergo a reverse process called mesenchymal–epithelial transition (MET) to continue their differentiation paths (Thiery et al. 2009; Lim and Thiery 2012). In many instances, the identification of an epithelial versus a mesenchymal state can be relatively fluid, and a partial EMT/MET frequently occurs to fulfill unique developmental tasks. These dynamic EMT/MET events highlight the enormous flexibility of presumably differentiated cells during morphogenesis.

In the past decade, an increasing number of studies have provided strong evidence for the reinitiation of the EMT developmental program in carcinoma progression and metastasis. This EMT program in tumor metastasis possesses many morphological and molecular features similar to those of the developmental EMT program. Importantly, due to the heterogeneity and constantly evolving microenvironment in human tumors, the EMT program in metastasis adapts to these conditions to allow tumor cells to successfully metastasize.

While EMT has been accepted as a critical program during embryogenesis, its role in carcinoma metastasis has been under debate (Tarin et al. 2005; Thomson et al. 2005; Garber 2008; Ledford 2011; Chui 2013). Many cell culture and mouse tumor model studies have clearly demonstrated the importance of EMT in tumor progression. However, the EMT process in human cancer, if present, remains difficult to identify, since carcinoma cells undergoing EMT share many similar morphological and molecular features with surrounding stromal fibroblasts. Furthermore, although primary tumors and circulating tumor cells (CTCs) present EMT features, distant metastases are generally epithelial in morphology, suggesting that,

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if functional, the EMT program is likely to be dynamically regulated during metastasis. In 2002, Jean Paul Thiery (2002), proposed the reversible EMT metastasis model in which primary epithelial tumor cells activate EMT to invade and disseminate throughout the body, while, upon arriving at distant sites, disseminated tumor cells (DTCs) undergo a reversion process, or MET, to form epithelial metastases. This intriguing hypothesis has brought EMT research to the forefront of carcinoma metastasis study.

The molecular program of EMT

Given that a number of recent reviews have focused on the molecular pathways regulating EMT/MET (Thiery et al. 2009; De Craene and Berx 2013; Zheng and Kang 2013), this section aims to provide an overview of the cellular and molecular definition for EMT/MET and establish a foundation for detailed discussions of these pathways in the context of tumor metastasis. The complex morphological and cellular changes during EMT require the cooperation of a large number of molecular signaling pathways and regulators. Based on their functionalities during EMT, we categorize them into three groups: the effector molecules that execute the EMT program (EMT effectors), the transcription factors that orchestrate the EMT program (EMT core regulators), and the extracellular cues that activate the EMT program (EMT inducers).

EMT effectors

Many of the hallmark EMT effector molecules are subcellular structure proteins that demarcate the epithelial or mesenchymal identity of a cell. During EMT, key molecular components of these structures are subjected to various levels of regulation. For example, the genes encoding various epithelial junction proteins, such as E-cadherin, α-catenin, and γ-catenin, are down-regulated at the mRNA and protein levels. Among them, E-cadherin is regarded as a gatekeeper of the epithelial state in various epithelial cell types. During EMT, E-cadherin gene transcriptional repression (Batlle et al. 2000; Cano et al. 2000; Hajra et al. 2002), promoter methylation (Graff et al. 1995; Kanai et al. 1997; Saito et al. 1998), and protein phosphorylation and degradation (Zhou et al. 2004; Bachelder et al. 2005; Lester et al. 2007) have all been observed in response to various inducing signals. Furthermore, intermediate filaments are shown to switch from cytokeratin to vimentin during EMT. Increased vimentin levels are also a consistent marker during various EMT events, while cytokeratin subtype switches tend to be variable and tissue type-specific.

Some additional key EMT effector molecules are proteins that promote cell migration and invasion during EMT. Fibronectin, an extracellular protein required for mesenchymal cell migration, is frequently induced upon activation of EMT. To promote cellular invasion through the ECM during EMT, a PDGF/PDGF receptor (PDGFR) autocrine loop is activated to promote invadopodia-mediated ECM degradation (Eckert et al. 2011). A number of non-epithelial cadherins, such as N-cadherin, and cell surface proteins, such as CD44 (Kuo et al. 2009) and integrin β6 (Bates et al. 2005), are induced and thought to be critical for proper cell migration. All of these proteins have been used to define the occurrence of EMT in tumors.

EMT core regulators

Execution of the EMT program involves the transcriptional alteration of many genes regulating cell adhesion, mesenchymal differentiation, cell migration, and invasion. In general, three core groups of transcriptional regulators have been consistently shown to be critical during various EMT events, thus being regarded as the core EMT regulators.

The first group is the transcription factors of the Snail zinc finger family, including Snail1 and Snail2, both of which are capable of directly binding to the E-boxes of the *E-cadherin* promoter to repress its transcription (Batlle et al. 2000; Cano et al. 2000; Hajra et al. 2002). The second group is the distantly related zinc finger E-box-binding homeobox family proteins Zeb1 and Zeb2, which are also able to suppress *E-cadherin* transcription (Comijn et al. 2001; Eger et al. 2005) via a double-negative feedback loop controlling Zeb1/Zeb2 and miRNA-200 family expression (Christoffersen et al. 2007; Bracken et al. 2008; Burk et al. 2008; Gregory et al. 2008; Korpal and Kang 2008; Korpal et al. 2008; Park et al. 2008; Kim et al. 2011b). Both the Snail and Zeb families of transcription factors have also been shown to repress the expression of other cellular junction proteins, such as claudins and ZO-1 (Ohkubo and Ozawa 2004; Vandewalle et al. 2005). The third group is the basic helix–loop–helix (bHLH) family of transcription factors, including Twist1 (Yang et al. 2004), Twist2 (Fang et al. 2011), and E12/E47 (Perez-Moreno et al. 2001), all of which can induce EMT alone or cooperatively. For example, Twist1 can not only repress *E-cadherin* through induction of Snail transcription factors (Li et al. 1995; Yang et al. 2004; Casas et al. 2011) but also activate programs associated with tumor invasion (Eckert et al. 2011), thus coordinating two major aspects of the EMT program.

EMT inducers

During tumor progression, EMT induction in tumor cells has not been associated with genetic alterations of the EMT core transcription factors, perhaps due to their essential roles in embryonic morphogenesis. Instead, carcinoma cells are thought to undergo EMT in response to a combination of extracellular signals in the tumor microenvironment. Many EMT-inducing signals tend to be cell type- or tissue type-specific and probably require the cooperation between multiple pathways. All major developmental signaling pathways, including TGF-β, Wnt, Notch, and growth factor receptor signaling cascades, have been implicated in some aspect of the EMT program. Most notably, the TGF-β pathway appears to be a primary inducer of EMT (Katsuno et al. 2013). For example, TGF-β and BMPs have been shown to induce the EMT core transcription factors Snail1/2, Zeb1/2, and

Twist1 (Thiery et al. 2009; Eckert et al. 2011). Interestingly, the ability of TGF-β/Smad signaling to induce EMT depends on the cooperation of several other pathways, including activation of the Ras kinase cascade via activated receptor tyrosine kinases (RTKs) or Ras mutations (Grunert et al. 2003) and cooperation from the Wnt/β-catenin/LEF-1 signaling pathway (Nawshad et al. 2005). One of the major sources of TGF-β in tumors is the stromal fibroblast cells in the tumor microenvironment (Hanahan and Weinberg 2011).

In addition to growth factor signaling, inflammatory cytokines and hypoxia in the tumor microenvironment have also been shown to promote EMT. The inflammatory cytokine TNFα can stabilize Snail1 via NF-κB activation (Wu et al. 2009) and induce Twist1 expression via IKK-β and NF-κB p65 activation (Li et al. 2012). Cytokines in the tumor microenvironment can also activate Stat3 via JAK kinases to induce Twist1 expression (Lo et al. 2007; Cheng et al. 2008). Hypoxic responses mediated by HIF-1 were also shown to induce the expression of Twist1 and Snail1 to promote EMT (Yang et al. 2008; Mak et al. 2010). Together, these studies indicate that extracellular cues from the tumor microenvironment play a critical role in activating EMT.

In summary, the EMT program involves a large number of cellular and molecular alterations. Since EMT-inducing signals are diverse and often context-dependent, EMT effectors and core transcription regulators are most widely used as molecular markers of EMT in human cancers. Further analysis of how individual EMT-inducing signals impinge on the EMT core regulators and effectors will provide a more comprehensive inventory of key players in EMT.

EMT/MET in tumor metastasis

The metastatic process is thought to consist of several steps. The initial escape from the primary site requires the epithelial tumor cells to become motile and degrade the underlying basement membrane and ECM; breakdown of these barriers initiates invasion into the nearby tissue parenchyma (step I: invasion). The next step of metastasis is termed intravasation, during which tumor cells invade across the endothelial lamina, penetrate into the blood or lymphatic vessels, and thereby enter the systemic circulation (step II: intravasation). Once in the circulation, only a small number of the disseminated neoplastic cells appear to be capable of surviving various insults within the circulation (step III: systemic transport). Eventually, some of the surviving cells may arrest in the vascular lumen and extravasate through the capillary endothelium into the parenchyma of distant organs (step IV: extravasation). In the new stromal environment, an even smaller subset of tumor cells establish micrometastases with the potential to proliferate into fully malignant, secondary tumors that are clinically detectable and eventually life-threatening (step V: colonization) (Thiery 2002; Fidler 2003; Kalluri and Weinberg 2009).

To clearly define the role of EMT in metastasis, we discuss both experimental and clinical evidence of EMT and its reversion program, MET, during the appropriate individual steps of tumor metastasis (Fig. 1).

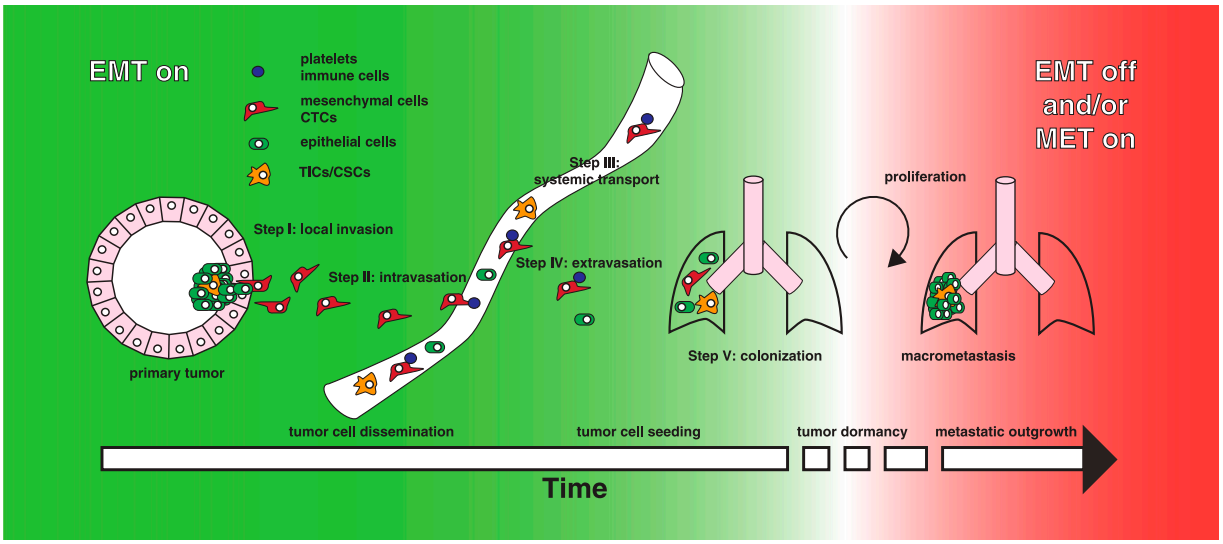

Figure 1. Model for reversible EMT over time. Epithelial cells undergo genetic transformation to become carcinoma in situ. Microenvironmental and genetic factors can promote the malignant conversion of these cells to activate the EMT program. During these early stages of tumor development, tumor cells that have undergone EMT can invade the local matrix (step I) and intravasate into the vasculature (step II). These epithelial–mesenchymal-transitioned cells are then transported in the circulation and survive via various prosurvival mechanisms (step III). At the distant tissue site, maintenance of the EMT program is required to help tumor cells extravasate into the parenchyma (step IV) to establish micrometastases. This initial seeding of tumor cells at distant sites can occur rapidly, after which cells may remain "dormant" for a long period of time. Subsequent colonization in distant organs requires the reversion of EMT and/or activation of the MET program to establish secondary tumors (step V).

Step I: EMT in malignant conversion and local invasion

Malignant conversion

Tumor initiation is often characterized by both genetic changes intrinsic to the tumor cells and alterations in the local microenvironment that promote tumor progression. Activation of the EMT program is classically thought to be a late stage event in malignant cancer to promote metastasis. However, several studies have implicated a possible role of EMT core transcription factors in the initial malignant transformation. Expression of Twist1 mRNA was detected in atypical ductal hyperplasia, a very early stage of primary breast tumor development in the MMTV-Neu mouse tumor model (Husemann et al. 2008). Twist1 was also shown to override oncogene-induced senescence and apoptosis by binding to p53 and promoting its degradation (Valsesia-Wittmann et al. 2004; Ansieau et al. 2008; Lee and Bar-Sagi 2010; Piccinin et al. 2012). By inhibiting p53, Twist1 was able to cooperate with oncogenes such as Her2 and H-ras to promote malignant transformation (Valsesia-Wittmann et al. 2004; Ansieau et al. 2008; Morel et al. 2012; Piccinin et al. 2012), suggesting a potential role of EMT genes in tumor initiation.

Activation of EMT is considered essential to allow carcinoma cells to lose cell–cell junctions and dissociate from each other for single-cell migration and invasion. For example, TGF-β pathway activation in EpH4-Ras mouse mammary carcinoma cells resulted in the loss of E-cadherin-mediated adherens junctions and gain of mesenchymal markers in cell culture and mouse tumor xenografts (Oft et al. 1996, 1998; Janda et al. 2002). Intercrossing the mouse pancreatic β-cell tumor (RIP-Tag2) model with transgenic mice that maintain E-cadherin expression in β-cell tumors arrested tumor development at the early adenoma stage, whereas expression of a dominant-negative form of E-cadherin induced early invasion and metastasis (Perl et al. 1998). Furthermore, mice carrying a genetic deletion of the *E-cadherin* gene on a mammary-specific p53-null background developed invasive lobular carcinomas, a subtype of breast cancer that presents individual migrating tumor cells (Derksen et al. 2006). Together, these studies strongly support a role of EMT in promoting single-cell invasion in primary tumors.

Degradation of the ECM

Carcinoma invasion requires tumor cells to gain the ability to degrade the underlying basement membrane and ECM. The EMT program is involved in this process through up-regulation of various matrix degradation enzymes by the EMT core regulators. Snail1 expression in MDCK epithelial cells and MCF-7 breast carcinoma cells increased MT1-MMP, MT2-MMP, and MMP9 expression (Olmeda et al. 2007a; Ota et al. 2009) and facilitated the breakdown of the basement membrane (Hotary et al. 2006; Ota et al. 2009). Conversely, Snail1 inhibition in epidermal and breast carcinoma cells decreased MMP9 expression and tumor growth and metastasis (Olmeda et al. 2007a,b). Consistent with these results, Snail2 was found to play an essential role in regulating tumor metastasis through induction of MT4-MMP and MMP2 (Shih et al. 2005; Huang et al. 2009). Interestingly, proteases have also been implicated in activating EMT by disrupting cell–cell junctions. Radisky et al. (2005) showed that MMP3 induces expression of Rac1, which increases reactive oxygen species (ROS) production and Snail1 expression to promote EMT (Orlichenko and Radisky 2008). Together, these results suggest a possible interplay between the loss of cellular junctions and the induction of proteases during EMT to promote tumor invasion.

More recently, there is emerging evidence that EMT transcription factors can also induce the formation of specialized subcellular structures called invadopodia to invade local ECMs (Eckert et al. 2011; Murphy and Courtneidge 2011). Invadopodia are actin-based protrusions that recruit various proteases such as membrane-tethered proteases (MT-MMPs), ADAMs, and MMPs, etc. to cell–matrix contact points to degrade ECM (Murphy and Courtneidge 2011). The EMT core transcription factor Twist1 was found to promote invadopodia formation through induction of PDGFRα expression and Src activation (Eckert et al. 2011). TGF-β was also shown to induce invadopodia formation in EpH4 and MCF10A mammary epithelial cells through up-regulation of Twist1 (Eckert et al. 2011) and the focal adhesion protein Hic-5 (Pignatelli et al. 2012) to promote matrix degradation and invasion. Furthermore, Zeppo1, a novel metastasis promoter that can repress E-cadherin expression, was found to induce EpH4.9 cells to form invadopodia-like structures in three dimensions (Slorach et al. 2011). Together, these studies suggest that the EMT program not only allows carcinoma cells to dissociate from each other but also provides them the ability to degrade ECM for single-cell invasion to initiate the metastatic cascade (Fig. 2).

Clinical evidence of EMT in primary tumor invasion

In the past, identification of carcinoma cells undergoing EMT in human tumor tissues has largely relied on histological analysis of E-cadherin expression. A partial loss of E-cadherin is associated with carcinoma progression and poor prognosis in various human tumor types, consistent with the role of E-cadherin as a caretaker of the epithelial state in carcinomas (Hirohashi 1998; Vincent-Salomon and Thiery 2003). In a few carcinoma subtypes, E-cadherin is lost at an early stage of the disease, so the tumor types present a permanent EMT phenotype. For example, a portion of lobular breast carcinomas (Berx et al. 1995, 1998) and diffuse gastric cancers (Becker et al. 1994; Oda et al. 1994) contain *E-cadherin* gene nonsense or frameshift mutations, while transcriptional suppression or post-translational modification of E-cadherin also contributes to the lack of E-cadherin expression in these tumors (Droufakou et al. 2001). Furthermore, in many late stage human carcinomas, E-cadherin expression appears to be heterogeneous, with E-cadherin-negative tumor cells interspersed within foci of E-cadherin-positive areas in the tumor (Bukholm et al. 1998, 2000), suggesting that some carcinoma cells might have undergone EMT.

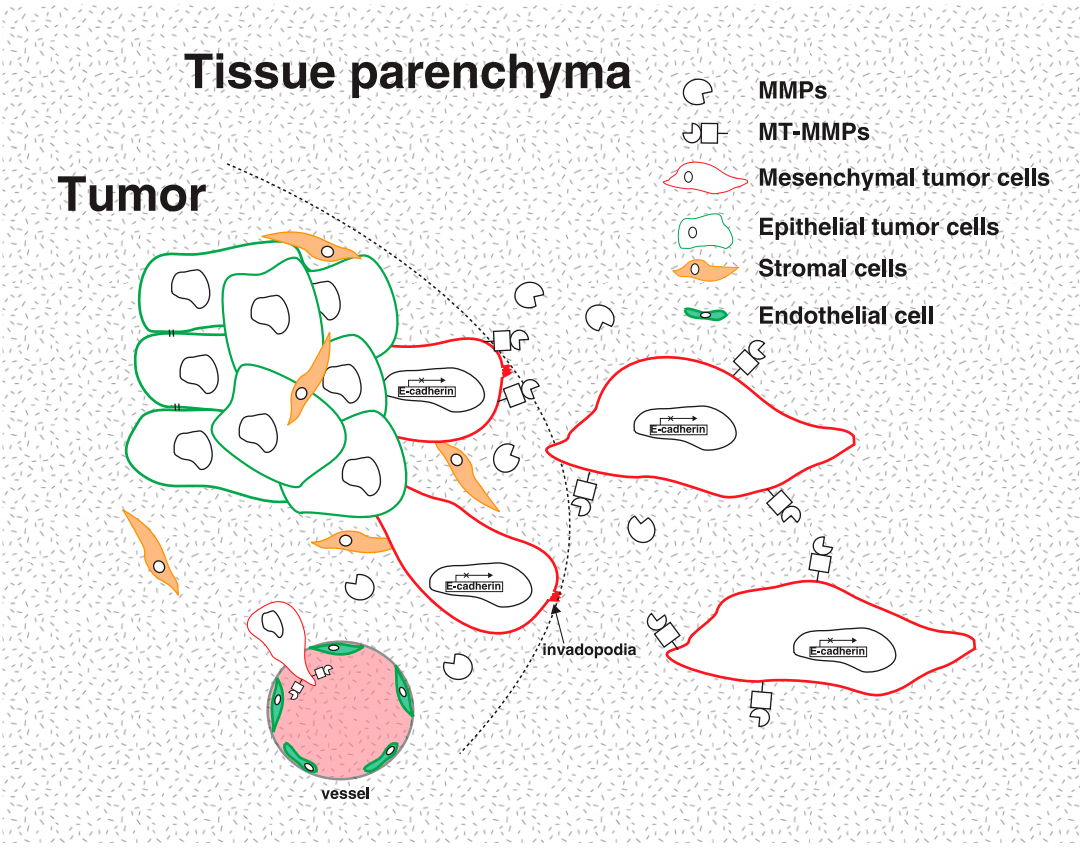

Figure 2. EMT in local invasion and intravasation. Activation of the EMT program is mostly characterized by the loss of E-cadherin expression. In order to invade through local basement membrane (surrounding the tumor or the tumor vasculature), these mesenchymal tumor cells up-regulate several secreted (MMPs) and membrane-tethered (MT-MMPs) proteases to break down ECM components. In addition, EMT factors can up-regulate specialized cellular structures such as invadopodia to promote local invasion. Expression of proteases can further induce EMT by breaking down cell–cell junctions, resulting in a positive feedback loop during malignant transformation of these cells.

Recent progress in gene expression profiling has shed more light on the prevalence of EMT in human cancer. Most notably, microarray analysis classified a claudin-low subtype of breast ductal carcinoma with low expression of E-cadherin and found that this subtype was enriched with an EMT gene signature including EMT core transcription factors Snail1, Twist1/2, and Zeb2 (Prat et al. 2010). In addition, immunohistochemical analysis of 28 molecular markers in 479 invasive breast carcinomas revealed clustering of EMT markers in tumors with a basal-like phenotype (Sarrio et al. 2008). Further analysis of these invasive basal/triple-negative subgroups found tumor cells that are double-positive for keratin and vimentin at the invasive front (Sorlie et al. 2001; Livasy et al. 2006; Rakha et al. 2006; Bonnomet et al. 2012). In colorectal carcinoma, ZEB1- and Snail1-positive cells with a mesenchymal morphology at the invasive front showed strong nuclear β-catenin signals (due to an APC mutation), suggesting that colon carcinoma cells, but not stromal cells, have undergone EMT (Brabletz et al. 2001, 2005; Spaderna et al. 2006). Recently, Celesti et al. (2013) performed Twist1 immunostaining in combination with fluorescent in situ hybridization (FISH) analysis of chromosome translocation unique to human colorectal tumor cells and found that 17 out of 20 human colon tumors contained Twist1$^+$ tumor cells with a mesenchymal phenotype. Together, these studies provide convincing clinical evidence of the occurrence of EMT (or at least partial EMT) in primary human carcinomas.

Step II: EMT in tumor cell intravasation

Following local invasion, tumor cells need to undergo an intravasation process to enter into the vasculature (lymphatic or blood vessels) for systemic dissemination. The precise mechanism of how tumor cells cross the endothelial barrier is largely unknown. Because of their size, tumors cells may require additional machinery to intravasate, unlike the smaller leukocytes that rely on diapedesis to migrate between endothelial cell (EC) junctions (Miles et al. 2008). The EMT program is thought to modulate the migratory and invasive properties of carcinoma cells to promote entry into the vasculature.

Technological advances in transendothelial assays, chick chorioallantoic membrane (CAM) assays, and intravital live imaging have facilitated the investigation of

EMT during the intravasation process. Using a transendothelial migration assay, Drake et al. (2009) found that Zeb1 expression in PC-3 human prostate cancer cells was required for enhanced migration through the EC barrier and increased metastatic colonization. Using a modified CAM assay that allows visualization of chorionic epithelium-derived vascular basement membrane, Ota et al. (2009) found that MCF-7 breast cancer cells expressing Snail1 could transmigrate through the underlying chorionic basement membrane and intravasate into the host vasculature. More interestingly, they showed that overexpression of Snail1 promoted intravasation through activation of membrane-bound MT1-MMP and MT2-MMP but not secreted MMPs, suggesting that direct contact between tumor cells and the endothelium is likely to be required for intravasation (Ota et al. 2009). Although further advances in in vivo imaging techniques are needed to carefully investigate the mechanisms regulating intravasation, these studies support a role of EMT in promoting carcinoma cells to breach through endothelium during intravasation (Fig. 2).

Step III: EMT in systemic transport

Upon entering systemic circulation, surviving tumor cells must possess the proper machinery to survive anoikis and then attach onto the blood vessel wall to prepare for extravasation from the circulation. Many recent studies in human cancer patients and mouse tumor models have identified the presence of EMT molecular markers in CTCs using various methods, including immunostaining, in situ hybridization, RNA sequencing, real-time quantitative PCR, and expression analysis (Yu et al. 2011). In general, most studies detected expression of EMT markers in CTCs; however, the functional significance of EMT in CTCs still awaits further evaluation.

Experimental evidence for EMT in CTCs

Currently, only a limited number of reports using mouse tumor models directly examine the involvement of EMT in producing CTCs. This may be due in part to the challenges in capturing CTCs that have fully undergone EMT, given that current isolation methods rely primarily on using epithelial markers, such as EpCam, to capture CTCs from the blood or bone marrow (Yu et al. 2011). To investigate tumor cell dissemination in vivo, Husemann et al. (2008) used the MMTV-Her2 mouse mammary tumor model and found increased Twist1 expression in hyperplastic lesions during early primary tumor development. Concomitantly, these mice presented increased DTCs in the bone marrow at this stage, suggesting that EMT may be partially responsible for the induction of CTCs/DTCs (Husemann et al. 2008). Consistent with these findings, in a K-Ras-driven mouse pancreatic tumor model, Rhim et al. (2012) detected circulating pancreatic tumor cells at the premalignant stage of tumor progression. The majority of these CTCs presented a mesenchymal phenotype and expressed Zeb2, indicating activation of the EMT program in these cells (Rhim et al. 2012). To demonstrate that activation of EMT directly promotes the production of CTCs, Tsai et al. (2012) examined the number of CTCs in a squamous cell carcinoma mouse tumor model in response to Twist1 induction. Indeed, Twist1 induction dramatically increased the number of CTCs compared with control mice, and these CTCs presented an EMT phenotype with loss of E-cadherin and expression of vimentin (Tsai et al. 2012). In the MDA-MB-468 breast tumor xenograft model, expression of Snail1 and Snail2 also increased the presence of vimentin-positive CTCs (Bonnomet et al. 2012). In these experimental studies, an increase in CTCs was associated with an increase in metastasis incidence, suggesting that EMT-induced CTCs directly contribute to effective metastasis formation.

Recent insightful studies have also revealed a potential mechanism for maintaining the mesenchymal state of CTCs. Labelle et al. (2011) found that CTCs were preferentially associated with platelet cells, which are a major source of TGF-β production in the blood. Importantly, depletion of platelets or inhibition of TGF-β secretion solely in platelets drastically reduced distant metastases (Labelle et al. 2011). Consistent with these experimental data, mesenchymal CTCs isolated from breast cancer patients were found clustered with platelet cells, and gene expression profiling of these CTCs found an enrichment of the TGF-β pathway (Yu et al. 2013). Together, these studies suggest that successful metastasis may depend on the maintenance of a mesenchymal state in CTCs.

One potential hypothesis for why CTCs need to maintain the EMT program is that EMT can prevent single tumor cells from detachment-induced anoikis while in circulation. For example, CTC survival may be aided by microtubule-based membrane protrusions called microtentacles that are thought to allow CTC aggregation and/or cell attachment to the blood wall. These structures have previously been shown to form in detached breast tumor circulating cells (Matrone et al. 2010a,b; Whipple et al. 2010). Expression of Twist1 or Snail1 in human mammary epithelial cells promotes microtentacle formation in detached cells, suggesting that EMT could aid CTC survival via microtenacle-based attachment of CTCs to leukocytes, platelets, and endothelium (Fig. 3; Matrone et al. 2010a).

Clinical evidence for EMT in CTCs

Many recent clinical studies have highlighted the use of CTCs as a prognostic marker for cancer progression and an indicator of therapeutic response. Studies in patients with metastatic breast and colorectal cancer showed that CTCs serve as an independent predictor of progression-free survival and overall survival (Christoffersen et al. 2007; Cohen et al. 2008). Interestingly, many clinical studies also detected the presence of EMT molecular markers in the CTCs. For example, CTCs from breast cancer patients showed reduced expression of epithelial markers and/or increased mesenchymal markers (Aktas et al. 2009; Kallergi et al. 2011; Raimondi et al. 2011;

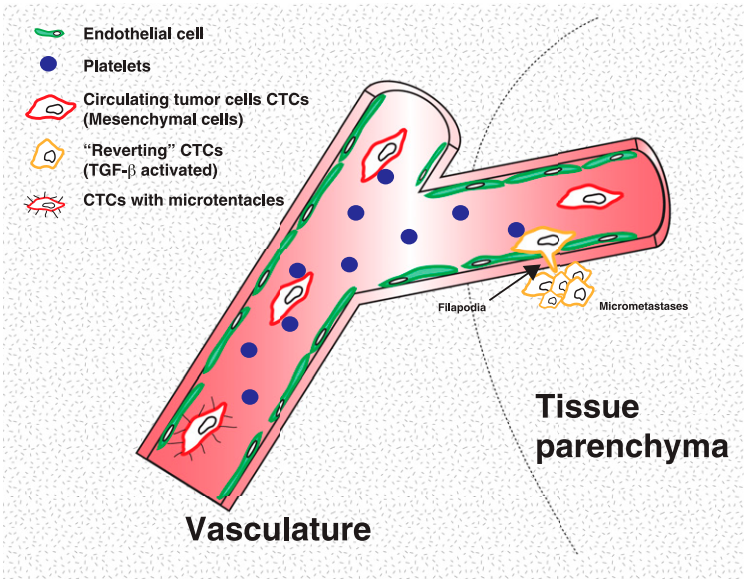

Figure 3. EMT in systemic transport and extravasation. Experimental and clinical samples have revealed an EMT signature in CTCs. This signature provides a possible biomarker to monitor tumor progression and/or therapeutic response. Experimental evidence suggests that platelets play a critical role in maintaining EMT activation in CTCs by providing the TGF-β signal. Furthermore, studies suggest that activation of EMT promotes microtentacle formation that allows tumor cell attachment to the endothelium and promotes cell survival. In order to extravasate at distant sites, tumor cells maintain an EMT phenotype and express cellular protrusions that allow extravasation, which is mediated by β1 integrin signaling.

Mego et al. 2012). Expression of Twist1 in DTCs isolated from bone marrows of breast cancer patients was correlated with early distant relapse (Watson et al. 2007). Using a similar approach, CTCs isolated from hepatocellular carcinoma (HCC) patients with metastasis expressed almost 20 times more Snail1 transcripts when compared with patients with no metastasis, demonstrating a potential functional role of Snail1 in HCC metastasis (Min et al. 2009). A recent study by Yu et al. (2013) quantified the proportions of CTCs with epithelial or mesenchymal phenotypes and found an association of mesenchymal CTCs with disease progression. One caveat of many studies involving CTCs is that, as previously discussed, the current CTC isolation methods rely primarily on using epithelial markers to capture CTCs. Thus, CTCs in the mesenchymal state may be missed during isolation (Paterlini-Brechot and Benali 2007; Pantel and Alix-Panabieres 2010; Kang and Pantel 2013). New technical advances are required to explore the potential utility of CTCs in both monitoring therapeutic responses and predicting cancer patient survival.

Step IV: EMT in tumor cell extravasation

Many classic studies of EMT regulators have used experimental metastasis assays, such as tail vein injection and intracardiac injection, to investigate tumor cell extravasation in the organ of interest. Studies have suggested that extravasation is a relatively rapid process (i.e., tumor cells extravasate 1–2 d following tail vein injection) (Cameron et al. 2000; Mendoza et al. 2010). However, tail vein assays involve injecting high numbers of tumor cells directly into the circulation. These injected tumor cells arrive at the lung microvasculature in such a large quantity that this process often results in intravessel growth rather than the sequential step of tumor growth in the tissue parenchyma following tumor cell extravasation. Thus, experimental models that more closely mimic physiological dissemination are needed to analyze the extravasation step.

Recently, new experimental systems have been developed to investigate how EMT is involved in the extravasation step. Stoletov et al. (2010) established an extravasation assay in zebrafish, which are optically transparent and allow real-time imaging of cell movement. Using this elegant system, they found that Twist1 expression in breast tumor cells promoted tumor cell extravasation through a β1 integrin-independent mechanism. Furthermore, they showed that Twist1-expressing cells formed large dynamic membrane protrusions during extravasation (Stoletov et al. 2010). Interestingly, Shibue et al. (2012) found that upon arriving at the distant tissue parenchyma, tumor cells present filopodium-like protrusions (FLPs) that contain integrin β1 to interact with the ECM. Not only was FLP formation found to be essential for successful metastasis, but the ability of various breast tumor cells to generate FLPs was correlated with their mesenchymal states, and their formation can be induced by the expression of Twist1 and Snail1 (Shibue et al. 2012). Together, these data indicate that the EMT program may promote extravasation and the initial lodging of tumor cells in distant organs (Fig. 3).

Step V: Reversion of EMT in metastatic colonization

As discussed, primary carcinomas and CTCs show strong cellular and molecular signatures of EMT; in contrast, resulting macrometastases are largely epithelial, which suggests that the involvement of EMT during metastasis is likely to be dynamic. Indeed, Chao et al. (2010) showed that tail vein injection of mesenchymal MDA-MB-231 cells into the secondary organ environment resulted in re-expression of E-cadherin through the passive loss of methylation in the *E-cadherin* promoter. Using vimentin as the mesenchymal marker, Bonnomet et al. (2012) found that primary MDA-MB-468 tumor xenografts and

the resulting lung metastases showed a heterogeneous expression pattern of vimentin, while CTCs expressed high levels of vimentin, Snail1, and Snail2. This suggests that vimentin-positive CTCs might have undergone MET to form vimentin-negative macrometastasis. Two recent studies have provided concrete experimental data to support such epithelial–mesenchymal plasticity during tumor metastasis. Using an inducible Twist1 mouse bearing skin tumors, it has been demonstrated that activation of EMT promotes the early steps of metastasis, including local invasion, intravasation, and extravasation. However, the loss of an EMT-inducing signal at the distant site was essential for cell proliferation and macrometastasis formation (Tsai et al. 2012). Consistent with these studies, Ocana et al. (2012) showed that downregulation of a novel EMT inducer, Prrx1, in BT549 human breast cancer cells was required for lung metastasis colonization upon tail vein injection. Specifically, Prrx1 cooperated with Twist1 to promote a more invasive phenotype, while loss of Prrx1 was required to revert EMT (Ocana et al. 2012). Together, these studies strongly argue that reversion of EMT is essential for metastasis colonization.

Why do tumor cells need to revert to an epithelial state to grow into macrometastases? Studies in cell culture showed that induction of EMT by Snail1 and Zeb2 directly represses cell division by inhibiting Cyclin D activity (Vega et al. 2004; Mejlvang et al. 2007). In an in vivo skin tumor model, activation of Twist1 was found to be associated with reduced tumor cell proliferation (Tsai et al. 2012). Since colonization demands tumor cells to restart proliferation upon extravasation into a foreign microenvironment, reversion of EMT may be required to provide such growth advantage. While these studies suggest that the induction of proliferation likely plays a key role in the reversion of EMT during colonization, it remains unanswered which signaling pathways couple EMT with cell proliferation. Other studies suggest that EMT regulators might provide additional assistance for metastatic colonization. The miR-200 family members are negative regulators of the EMT inducer Zeb1 and vice versa (Christoffersen et al. 2007; Bracken et al. 2008; Burk et al. 2008; Gregory et al. 2008; Korpal and Kang 2008; Korpal et al. 2008; Park et al. 2008; Kim et al. 2011b). Interestingly, re-expression of miR-200 family members was shown to enhance colonization possibly by repressing Sec23a-mediated secretion of metastasis-suppressive proteins, including Igfbp4 and Tinagl1 (Fig. 4; Korpal et al. 2011).

Another unanswered question is how the EMT reversion process occurs in distant organs. In other words: Is the absence of an EMT-inducing signal sufficient for EMT reversion? Are additional MET-inducing signals required to actively promote MET? Recent studies indicate that both scenarios are possible in promoting EMT reversion. In a skin tumor model, the absence of a Twist1 signal (or the withdrawal of Twist1-activating signal) in DTCs resulted in macrometastasis formation (Tsai et al. 2012). However, this study does not exclude the possibility that additional MET-inducing signals may also contribute to

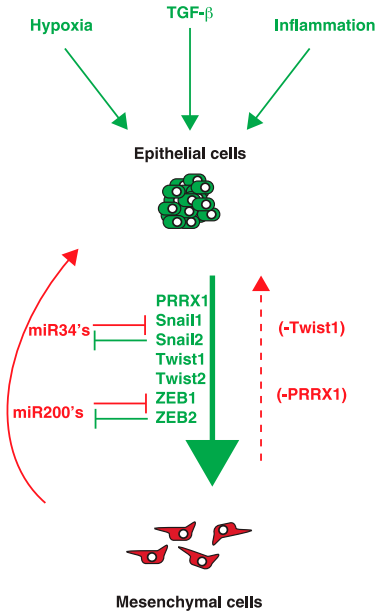

Figure 4. Mechanisms of EMT reversion. Colonization at distant sites requires the reversion of EMT to promote tumor cell proliferation. The interplay between EMT activators and inhibitors (i.e., MET activators) plays a critical role in metastatic outgrowth. The loss of EMT activators such as Twist1 or Prrx1 appears to be required to promote EMT reversion. However, signals from the microenvironment in distant sites may also shift the balance from EMT activators to EMT inhibitors or MET activators. It is unknown when or how these factors are regulated during tumor progression, which may impact treatment of metastatic disease.

colonization. Gao et al. (2012) showed that versican expression by myeloid cells in the lung metastatic niche promoted lung colonization by inducing MET, thus supporting the notion that signal inputs from the metastatic niche could regulate epithelial–mesenchymal plasticity in distant sites. Although it is currently unknown how microenvironmental signals regulate MET, EMT core transcription factors are considered primary targets for such regulation. For example, a number of EMT core transcription factors are negatively regulated by miRNAs, including miR-200 family members that regulate Zeb2 as well as miR-34 family members that regulate Snail1 and Zeb1. It is possible that microenvironmental signals could impinge on these miRNAs to turn off EMT at distant organs (Fig. 4; Kim et al. 2011a; Siemens et al. 2011). Given that micrometastasis outgrowth is a key rate-limiting step in metastasis, more studies on the molecular regulators of MET could shed light on therapeutic approaches to inhibit tumor colonization.

Adding to the complexity of metastatic colonization is the clinical observation that DTCs can remain "dormant" for many years before regrowth. These cells are thought to reside in the secondary tissue parenchyma as a micrometastatic lesion or in the bone marrow and mobilize to secondary sites prior to regrowth (Hedley and Chambers 2009). Due to the lack of specific molecular markers to detect and isolate dormant micrometa-

stases from epithelial organs in cancer patients and mouse tumor models, our current understanding of tumor dormancy is largely based on micrometastases isolated from the bone marrow. In one study, micrometastases from the bone marrow of breast cancer patients were isolated, and Twist1 expression was identified as a marker for early distant metastasis relapse (Watson et al. 2007). This result is consistent with a role of EMT in promoting tumor cell dissemination, as discussed earlier. More importantly, together with the notion that reversion of EMT is required for macrometastasis colonization, they suggest that the inability to revert EMT in DTCs might contribute to metastasis dormancy. Further studies using relevant experimental tumor models are needed to better evaluate the involvement of EMT in tumor cell dormancy.

While large numbers of studies have demonstrated a cell-autonomous role of EMT in promoting tumor cell dissemination, an alternative "cooperative" model proposes a supporting role for tumor cells that have undergone EMT to aid metastasis. By mixing uniquely labeled epithelial tumor cells and mesenchymal tumor cells to generate primary tumors, Tsuji et al. (2008) reported that epithelial cells require the cooperation of mesenchymal cells to lead the way for intravasation and CTC generation. However, only the epithelial tumor cells, but not the mesenchymal tumor cells, could generate macrometastases (Tsuji et al. 2008). In addition, the collaboration between tumor-initiating cells (TICs) and cells having undergone EMT accelerated metastatic colonization by the TICs (Celia-Terrassa et al. 2012). Both studies suggest that epithelial and mesenchymal tumor cells may cooperate to successfully form metastatic tumors (Tsuji et al. 2009). However, given that the mesenchymal tumor cells used in these studies have undergone a permanent EMT and cannot colonize in distant organs, it is plausible that the epithelial tumor cells still need to undergo a dynamic EMT/MET process to succeed in generating macrometastases.

Emerging frontiers of EMT

EMT and cancer stem cells (CSCs)

The role of CSCs or TICs in tumor progression has been at the forefront of cancer research in recent years (Nguyen et al. 2012). CSCs are generally characterized by their ability to initiate tumors in serial dilution transplantation assays. They express multiple cell surface markers, including $CD44^{high}$, $CD24^{low}$, and $CD133^{high}$, depending on the tumor type. Interestingly, a number of studies have demonstrated that EMT activation can generate CSCs or disseminated cells that have tumor-initiating properties. Studies by the Weinberg group (Mani et al. 2008) and the Puisieux group (Morel et al. 2008) showed that activation of EMT by TGF-β, Snail1, Twist1, and Zeb1 in normal human mammary epithelial cells can promote a CSC-like phenotype with tumor-initiating properties. In mouse mammary epithelial cells, Snail2 was found to be a critical player in regulating normal mammary stem cells (Guo et al. 2012). Twist1 was also found to be capable of suppressing CD24 expression, providing a direct connection between an EMT transcription factor and CSC generation (Vesuna et al. 2009). In breast cancer cells, activation of urokinase-type plasminogen activator receptor (uPAR) was found to reversibly activate EMT, and activation of this pathway was capable of generating CSC-like properties (Lester et al. 2007; Jo et al. 2009, 2010). More interestingly, recent studies also found that basal breast cancer non-CSCs are populations that can generate CSCs de novo, and the CSC plasticity is controlled by the chromatin state of the Zeb1 promoter (Chaffer et al. 2011), further highlighting the critical role of EMT regulators in regulating CSC plasticity during tumor progression.

Consistent with these experimental results, numerous studies have provided correlative evidence relating EMT to the emergence of a CSC phenotype in human cancers. In breast cancer patients, disseminated breast cancer cells from pleural effusions, which likely have undergone EMT, are enriched for a $CD44^{high}$, $CD24^{low}$ CSC-like population (Al-Hajj et al. 2003). In addition, stem cells isolated from normal breast tissue or breast cancers express a number of canonical EMT markers. Of clinical significance, studies have shown that the tumor immune response resulted in EMT-associated emergence of $CD44^{high}$–$CD24^{low}$ CSCs (Santisteban et al. 2009).

Despite the strong evidence of a pro-CSC-forming role by EMT core regulators, there are experimental data that implicate EMT as having a negative impact on TICs. Celia-Terrassa et al. (2012) showed that cancer cells with a pronounced epithelial phenotype were enriched with highly metastatic TICs, whereas the mesenchymal-like cells lacked TICs. Furthermore, forced expression of Snail1 in the phenotypically epithelial cells suppressed their self-renewal and metastatic capabilities, suggesting that EMT activation may in fact suppress the tumor-initiating properties of CSCs (Celia-Terrassa et al. 2012). These contradictory results could be due to the difference in additional genetic/epigenetic alterations in the individual cell types studied. A recent study found that removing an EMT regulator, Prrx1, was required for the tumor-initiating ability of breast cancer cells expressing Twist1 (Ocana et al. 2012). Future studies to clearly define the difference in signaling pathways regulating EMT versus TICs will provide much-needed information on how the EMT program and TIC regulation are intervened.

EMT and drug resistance

One major obstacle in cancer therapy is that cancer patients can develop resistance to treatment over time. An increasing number of reports suggests a potential role of EMT in conferring drug resistance. Studies using colorectal cell lines have shown that expression of EMT inducers Snail1 or TGF-β in SMAD4-null cells increased resistance to chemotherapy (Hoshino et al. 2009; Papageorgis et al. 2011). Conversely, colorectal cell lines that were rendered oxaliplatin-resistant showed phenotypic and gene expression changes consistent with EMT (Yang et al. 2006; Kawamoto et al. 2012). Interestingly,

tumor specimens taken from patients who had received 1 wk of preoperative chemotherapy prior to surgery resection displayed a more mesenchymal gene signature compared with prechemotherapy biopsy samples. Furthermore, recurrent tumors frequently exhibited an EMT gene signature (e.g., decreased E-cadherin and increased vimentin) (Kawamoto et al. 2012). Shintani et al. (2011) found that, in non-small-cell lung carcinoma (NSCLC) patients, tumor biopsy prior to chemotherapy treatment showed epithelial markers but that this phenotype shifted toward mesenchymal markers following treatment. Consequently, the disease-free survival rate was lower in patients whose tumors presented an EMT phenotype compared with EMT-negative tumors (Shintani et al. 2011).

Besides the evidence of drug resistance to chemotherapy, recent studies have also suggested a role of EMT in drug resistance toward targeted therapies. Using several human NSCLC lines, Thomson et al. (2005) demonstrated that cell lines expressing epithelial markers were more sensitive to epidermal growth factor receptor (EGFR) inhibition, whereas cells lines presenting mesenchymal markers were more resistant to treatment. In gefitinib-resistant PC9/AB2 lung cancer cells, Notch1 was found to promote EMT. Knockdown of Notch1 reverted the EMT phenotype and rendered these cells sensitive to gefitinib (Xie et al. 2012), implicating a strong correlation between EMT activation and drug resistance.

While it remains unknown whether and how the EMT program directly impinges on drug resistance, several possible mechanisms might be in play. First, since activation of EMT reduces cell proliferation (Vega et al. 2004; Mejlvang et al. 2007), slowing the cell cycle machinery will increase resistance to chemotherapy, which generally targets highly proliferative cells. Second, EMT core transcription factors, including TGF-β, Snail1, Snail2, and Twist1, have been shown to confer resistance to cell death via various pathways, most notably by antagonizing p53-mediated apoptosis (Inoue et al. 2002; Kajita et al. 2004; Vega et al. 2004; Gal et al. 2008), thus providing a potential survival advantage. Third, as discussed above, the EMT program can promote CSC properties. CSCs were found to be inherently more resistant to conventional cancer chemotherapies than rapidly proliferating progenitor cells and differentiated tumor cells. These CSCs were also found to be responsible for tumor reoccurrence and capable of establishing metastases (Phillips et al. 2006; Li et al. 2008). Indeed, conventional chemotherapy is shown to enrich CSCs in breast cancer patients. For example, mammospheres isolated from chemotherapy-treated breast tumors showed similar mammosphere-initiating capacity after eight passages in culture, whereas cells from untreated tumors vanished within three passages, suggesting again an increase in CSCs after chemotherapy (Yu et al. 2007). Li et al. (2008) further demonstrated that chemotherapy increased the number of $CD44^{high}$–$CD24^{low}$ cell population and that these cells have stem-like features. Together, these studies strongly indicate that activation of EMT contributes to cancer therapy resistance.

Future directions in therapeutic targeting of EMT/MET

Metastatic diseases are responsible for >90% of carcinoma-related deaths. Given the strong evidence supporting a critical role of EMT in tumor metastasis, targeting this process is thought to be a promising approach to treat invasive cancer. However, current treatment modalities remain limited in their efficacy in targeting cells undergoing EMT. This may be due in part to potential drug resistance in this population of cells (as discussed above) and the lack of appropriate targets in the core EMT program. Because EMT core transcription factors remain technically challenging to target, targeting the activation or the functional consequence of EMT is perhaps the more effective approach. Several groups have performed high-content drug screens to identify potential inhibitors of EMT in response to various EMT-inducing signals. Interestingly, the majority of the compounds obtained appear to be involved in inhibiting the specific EMT-inducing signals used in the screen. For example, rapamycin and 17-AGG were identified as inhibitors of TGFβ-induced EMT by modifying the TGFβ pathway (Reka et al. 2011), while inhibitors of ALK5, MEK, and SRC could interfere with EMT in response to EGF, HGF, and IGF-1 (Reka et al. 2011; Chua et al. 2012). Salinomycin was also identified as inducing selective killing of E-cadherin-null breast epithelial cells compared with E-cadherin-positive cells (Gupta et al. 2009), although its molecular action toward EMT is unknown.

The plasticity of EMT in metastasis provides another level of complexity regarding the appropriate time window to target EMT in patients. Several studies suggest that targeting the TGFβ pathway to inhibit EMT or blocking tumor cell invasion through inhibiting PDGR signaling may be appropriate as metastasis prevention strategies in early stage carcinomas. Once tumor cells have disseminated from the primary tumor, inhibiting EMT could be counterproductive, since reversion of EMT appears to be beneficial for disseminated carcinoma cells to regain proliferation and colonize distant organs (Tsai et al. 2012). Instead, the EMT features in CTCs and disseminated dormant tumor cells present several unique opportunities for therapeutic intervention. First, since DTCs present molecular markers of EMT, unique EMT surface markers expressed in these cells could be ideal targets for T-cell-based immunotherapy. Second, since an increasing number of studies suggest a role of EMT in promoting chemoresistance, combining chemotherapies with EMT inhibitors holds promise to overcome chemoresistance in dormant tumor cells, thus providing a unique therapeutic approach to eradicate dormant tumor cells. Last, preventing the reversion of the EMT program in dormant micrometastases would be a novel approach to prevent resurrection of dormant tumor cells. In the near future, improving our understanding of the molecular regulation of the dynamic EMT/MET programs during tumor metastasis will help to provide much-needed effective treatment to eradicate metastatic diseases.

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REVIEW

Invading one step at a time: the role of invadopodia in tumor metastasis

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The ability to degrade extracellular matrix is critical for tumor cells to invade and metastasize. Recent studies show that tumor cells use specialized actin-based membrane protrusions termed invadopodia to perform matrix degradation. Invadopodia provide an elegant way for tumor cells to precisely couple focal matrix degradation with directional movement. Here we discuss several key components and regulators of invadopodia that have been uniquely implicated in tumor invasion and metastasis. Furthermore, we discuss existing and new therapeutic opportunities to target invadopodia for anti-metastasis treatment.

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INTRODUCTION

Metastasis, the spread of tumor cells from a primary tumor to a secondary site, is a complex, multistep process, and is the main cause of mortality in cancer patients. During metastasis, carcinoma cells invade the surrounding extracellular matrix (ECM), intravasate through endothelium into the systemic circulation, then extravasate again through the capillary endothelium and finally establish secondary tumors at distant sites.[1] Several key stages of metastasis, including invasion, intravasation and extravasation, are thought to involve ECM degradation and remodeling. In recent years, actin-rich subcellular protrusions known as invadopodia have been shown to be critical for ECM degradation.[2] Invadopodia consist of an actin-rich core surrounded by a number of important protein components, including cytoskeletal modulators, adhesion proteins, scaffolding proteins and signaling molecules.[3] The central function of invadopodia is to recruit various matrix proteases to cell–ECM focal contacts for matrix degradation.

Unlike other actin-based protrusions such as lamellipodia and filopodia that are present in normal cells, invadopodia are uniquely present in invasive cancer cells and are considered as the transformed version of podosomes, which are present in highly invasive normal cells such as macrophages, osteoclasts and dendritic cells. In-depth reviews have covered all the molecular components of podosomes/invadopodia and their biological functions in development and pathogenesis.[3] This review focuses on a selective set of invadopodia components and regulators that are relatively unique to invadopodia and are specifically modulated in human cancers (Figure 1, Table 1). In addition, we will discuss the contributions of these invadopodia components to invasion and metastasis, and the therapeutic opportunities to target these components for cancer treatment.

STRUCTRUAL COMPONENTS

As an actin-based structure, invadopodia engage a large number of structural and regulatory proteins that control actin dynamics, such as Arp2/3, Enabled (Ena)/vasodilator-stimulated phosphoprotein (Vasp) and various small GTPases. Here we discuss three factors, cortactin, MENA and Tks proteins, that have critical roles at invadopodia and have been implicated in tumor progression. Cortactin and MENA are both key factors of actin polymerization and dynamics; therefore, their roles in tumor invasion and metastasis go beyond invadopodia to general cell migration and other actin-based cellular processes. In contrast, Tks proteins are known to be more specifically involved in invadopodia formation, therefore their impact on tumor invasion and metastasis is thought to be largely because of their functions at invadopodia.

Cortactin

Cortactin is a cytoskeletal protein that when phosphorylated can recruit the Arp2/3 complex to promote invadopodia formation. Cortactin was originally identified as a Src phosphorylation target in Src-transformed chicken embryo fibroblasts.[4] Src binds to cortactin through direct interaction with the SH2 domain of Src,[5] and phosphorylates cortactin in v-Src-transformed 3T3 fibroblasts.[4,6]

As Src kinase has an essential role in invadopodia regulation (discussed in detail in a later section), cortactin has been shown to be a key regulator of actin polymerization at invadopodia in response to Src activation. The association of cortactin with invadopodia was first described in MDA-MB-231 cells, in which microinjection of anti-cortactin antibodies reduced their ability to degrade ECM.[7] Furthermore, immunoprecipitation of cortactin revealed its presence in invadopodia-enriched membrane fractions.[7] Finally, immunofluorescence indicated cortactin at actively degrading invadopodia.[7,8] Knockdown of cortactin in MDA-MB-231 cells resulted in inhibition of actin/cortactin-positive puncta and matrix degradation, suggesting that cortactin is required for invadopodia formation and function.[8] Cortactin localization at invadopodia coincided with phosphotyrosine puncta, which is consistent with Src regulation of cortactin phosphorylation.[9] Src activation of cortactin resulted in the localization of Nck1 and N-WASP at invadopodia, in addition to the disengagement of cofilin, all of

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which are required for Arp2/3-mediated actin polymerization to promote invadopodia formation.[10,11]

As cortactin regulates various actin-based cellular programs, including invadopodia and lamellipodia formation and dynamics, its role in tumor cell invasion and metastasis is well documented. Overexpression of cortactin in NIH3T3 cells resulted in increased invasion in a Matrigel Boyden chamber assay.[12] Similarly, overexpression of cortactin in MDA-MB-231 cells resulted in increased invasion, which correlated with increased metastatic bone lesions.[13] In contrast, overexpression of a phosphorylation-deficient cortactin inhibited invasion and resulted in minimal bone metastatic lesions.[13] Similar results were obtained in a hepatocellular carcinoma model, where overexpression of wild-type cortactin in the non-metastatic hepatocellular carcinoma cell line KIM1 increased metastatic incidence without affecting primary tumor growth.[14]

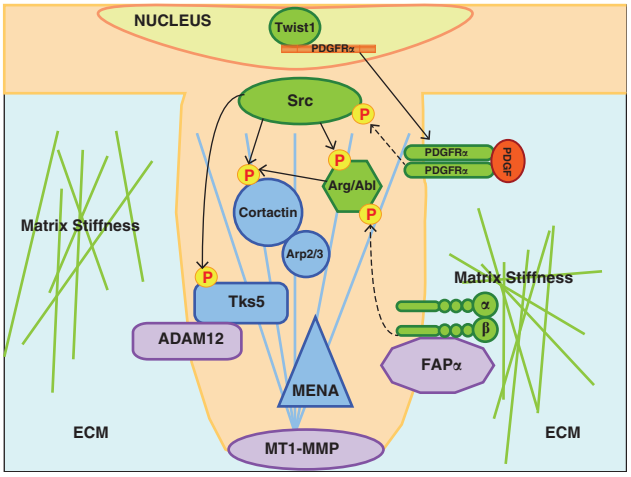

Figure 1. An overview of the invadopodia components and regulators discussed in this review. Twist1-induced expression of PDGFRα leads to increased Src kinase activity, which serves as a trigger for invadopodia formation. Src-mediated phosphorylation of the structural components cortactin and Tks5, and the Arg/Abl tyrosine kinase promotes invadopodia assembly. Integrin β1 serves as an adhesion mediator between invadopodia and ECM, an activator of Abl/Arg at invadopodia, a sensor of matrix stiffness to regulate invadopodia assembly and a potential docking site for FAPα. Structural components of invadopodia, which include the actin core, are labeled in blue, proteases are labeled in purple and regulatory components are labeled in green.

Cortactin was first implicated in the progression of human cancers through gene amplification at chromosome 11q in breast and squamous cell carcinomas.[13,15,16] In addition, high levels of cortactin expression are also observed in human ovarian, bladder and lung cancer.[4,7,13,15] Importantly, overexpression of cortactin is associated with poor patient prognosis in breast and head and neck squamous carcinomas, further highlighting a critical role of cortactin in human tumor progression.[13,16]

MENA

Mena is a member of the Enabled (Ena)/VASP family of proteins, which are involved in the regulation of actin polymerization. Mena protein is upregulated in breast, pancreatic, colon, gastric, cervical cancers and melanoma, with the expression of specific isoforms regulating the invasive properties of breast cancer cells.[17–19] Owing to its role in actin polymerization, Mena is a logical regulator of invadopodia formation. Mena was found to co-localize with cortactin and F-actin at invadopodia.[18] In addition, Mena-null mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) mammary tumors exhibited reduced invasion into the surrounding stroma.[20] In conjunction, Mena-null mice had significantly fewer circulating tumor cells and lung metastases, when compared with control mice, suggesting that Mena expression is necessary for tumor cell intravasation and invasion.[20]

Interestingly, gene profiling in rat MTLn3 and mouse PyMT breast tumors identified an invasion-specific isoform of Mena, called Mena^{INV}, whose expression was correlated with invasive ability.[17] Mena^{INV} increased invasion of MTLn3 cells into collagen gels, indicating a unique role for Mena^{INV} in carcinoma cell invasion.[18] In addition, MtLn3 cells expressing Mena^{INV} exhibited increased membrane protrusions compared with parental MTLn3 cells.[18] In terms of metastatic progression, Mena^{INV} expression was associated with highly metastatic carcinomas in the PyMT mouse mammary tumor model.[21] Philippar *et al.*[18] also observed that overexpression of Mena^{INV} in MTLn3 cells resulted in increased micrometastatic lung formation, despite no effect on primary tumor growth, again indicating the importance of regulating Mena mRNA splicing in tumor invasion and metastasis.[20]

Tks adaptor proteins

The Tks adaptor proteins Tks4 and Tks5 are named after tyrosine kinase substrate with 4 or 5 SH3 domains, respectively. Tks proteins only contain SH3 and PH domains for protein–protein and protein–lipid interactions; therefore they are thought to serve as adaptor proteins that recruit other proteins and lipids[22] for invadopodia assembly.

Table 1. A list of invadopodia components and regulators, their roles in invadopodia, invasion and metastasis, and their therapeutic potentials

Components	*Cancer*	*Invadopodia*	*Invasion*	*Metastasis*	*Therapeutics*
Core elements					
Cortactin	Breast, head and neck, lung, ovarian and bladder	7–11	12,13	13,14	—
MENA	Breast, pancreatic, colon, gastric, cervical cancer and melanoma	18	17–19,21	18,20,21	—
Tks	Breast, melanoma	23,27–29	24,29	24,30	—
Proteases					
MT1-MMP	Breast, lung, squamous cell, colorectal and melanoma	8,40,42	44–46	48,49	50–53
ADAM12	Breast, prostate, lung, brain, liver and bone	25,57	58,62	62	—
Serine proteases	Breast, colon, ovarian and melanoma	65,66	—	—	74,75,79
Regulatory components					
Src	Breast and colon	9,81–84	9,83–86	85,87–89,91	89,93–95
Abl kinases	Leukemia and breast	84,98	97,99,100	100	93,101,102
Integrin	—	103–106	103	109	—
Twist 1	—	24	24	111,112	—

The role of Tks5 in invadopodia was first discovered using a Src substrate screening assay, and further characterized based on its localization to invadopodia in Src-transformed fibroblasts.[23] Tks5 is required for both invadopodia formation and invasion activity in a variety of human cancer cell lines, as knocking down Tks5 reduced matrix degradation activity and invasion.[24] Likewise, introduction of Tks5 into the human breast epithelial cell line T47D, which lacks endogenous Tks5 expression, promoted invadopodia formation.[23] Tks5 was shown to bind to ADAM12 (A Disintegrin And Metalloproteinase),[25] a metalloproteinase associated with invadopodia. Furthermore, Tks5 is also associated with the actin regulatory protein N-WASP,[26] and involved in the recruitment of AFAPA-110, p190RhoGAP and cortactin to invadopodia.[27] Finally, Tks5 binds to $p22^{phox}$, a part of the NADPH oxidase complex that generates reactive oxygen species, that facilitates invadopodia assembly and function.[28]

The family member Tks4 also localizes to invadopodia in Src-transformed cells and is required for invadopodia assembly.[29] However, Tks4 and Tks5 seem to have non-redundant roles in invadopodia function. Cells lacking Tks4 formed actin puncta resembling invadopodia, but these cells failed to degrade ECM components, even in the presence of high levels of Tks5.[29] This is thought to be because of a crucial role of Tks4 in recruiting MT1-matrix metalloproteinase (MMP) to invadopodia, as no MT1-MMP was detected in the rudimentary invadopodia present in Tks4 knockdown cells.[29]

To test the role of Tks5 in tumor metastasis, Eckert *et al.*[24] showed that knocking down Tks5 in Ras-transformed human mammary epithelial cells that overexpress Twist1 inhibited both local invasion and the ability of these cells to form lung metastases, whereas primary tumor formation rates were not altered. These data strongly indicate that Tks5, and likely its role in invadopodia assembly, is required for the early steps of metastasis. To test whether Tks5 functions during extravasation and metastatic outgrowth, Blouw *et al.*[30] injected Src-transformed 3T3 cells with Tks5 knockdown into immunocompromised mice via tail vein. Although Tks5 knockdown did not significantly affect the number of lung colonies, the metastases derived from the cells with Tks5 knockdown were significantly smaller. These data suggest that Tks5 could be further required for the expansion of secondary tumors in distant sites.

PROTEASES

Given their central function to recruit proteases to cell–matrix contacts for matrix remodeling, invadopodia are shown to contain a large numbers of proteases. The proteases found at invadopodia include metalloproteases (both secreted and membrane-tethered MMP), the ADAM family members and membrane-bound serine proteases, all of which have been implicated in cancer progression and metastasis. Past research has focused on developing metalloproteinase inhibitors to suppress ECM degradation and tumor metastasis. Although these inhibitors show promising results in cell culture and tumor xenograft models, numerous metalloproteinase inhibitors have failed during clinical trials.[31] Further studies indicate that some metalloproteinases could have antitumorigenic effects.[32] Therefore, the strategy of broadly blocking metalloproteinases to abrogate metastasis might not be a viable approach to prevent tumor metastasis. Here we discuss a few proteases that are unique to invadopodia and might be promising new targets in inhibiting tumor invasion and metastasis.

Metalloproteinases

MT1-MMP. MT1-MMP (also known as MMP14), a membrane-anchored metalloproteinase, is considered a central factor of invadopodia-mediated ECM degradation. MT1-MMP cleaves[33] several substrates *in vitro*, including ECM components such as fibronectin, type I, II and III collagen, laminins, vitronectin and aggrecans.[34–37] In addition, MT1-MMP is capable of activating other MMP zymogens: MT1-MMP activates MMP2 by cleaving the N-terminal prodomain of pro-MMP2[38] and MMP9 is activated through an activation cascade involving MT1-MMP, MMP2 and MMP3.[39] MT1-MMP is shown to be required for the matrix degradation activity of invadopodia. Artym *et al.*[8] found that cortactin aggregation initiated accumulation of MT1-MMP at invadopodia. This study also found that although MT1-MMP knockdown moderately impacted the initial stages of invadopodia formation, matrix degradation was strongly suppressed,[8] indicating that MT1-MMP is essential for functional invadopodia.

MT1-MMP is delivered to invadopodia by multiple routes. Studies from Yu *et al.*[40] show that N-WASP, which promotes actin nucleation, promotes the delivery of MT1-MMP from late endosomes to invadopodia.[41] MT1-MMP can also be mobilized by the Rab8-dependent secretory pathway and delivered to collagen-contact sites.[42] Finally, MT1-MMP can also be internalized by both clathrin- and caveolae-mediated endocytosis,[3,43] and this internalization serves to recycle MT1-MMP back to invadopodia when needed.

A key inducer of invadopodia, Src kinase, has also been shown to directly regulate the delivery of MT1-MMP to invadopodia. Src-mediated phosophorylation of MT1-MMP in its AP2 clathrin adaptor binding domain slows endocytosis of MT1-MMP and increases matrix degradation activity.[43] In addition, phosphorylation of MT1-MMP by Src at Tyr573 has been shown to be required for tumor cell proliferation, invasion of three-dimensional (3D) collagen matrices and tumor growth in nude mice.[44,45] Finally, a recent study found that this phosphorylation was required for mono-ubiquitination of Lys581, which is involved in MT1-MMP trafficking to the cell surface and cellular invasion through collagen matrices.[46]

An increase in MT1-MMP expression is generally associated with poor prognosis in a wide variety of human cancers, including breast, lung, melanoma, colorectal and squamous cell carcinomas.[47] MT1-MMP expression has also been directly linked to metastasis in mouse tumor models. MT1-MMP-deficient mice were bred with MMTV-PyMT mice, and then PyMT-positive mammary glands lacking MT1-MMP were orthotopically transplanted into wild-type mice. Although palpable tumors developed faster with MT1-MMP-deficient mammary glands, metastatic spread was reduced by 50%.[48] Consistent with this study, Perentes *et al.*[49] injected MDA-MB-231 cells with MT1-MMP knockdown into the mammary fad pad of severe combined immunodeficiency (SCID) mice and found that MT1-MMP knockdown resulted in a significant decrease in lung metastasis without affecting primary tumor growth.[49] These results suggest that MT1-MMP is required for metastatic development *in vivo*.

As blocking MMP activity has failed in clinical trials as an anti-metastasis therapy, possibly due to the broad spectrum of inhibition and severe toxicities,[50,51] new therapeutic strategies aim to target the specific MMPs that contribute to disease progression.[50] A fully humanized monoclonal antibody (DX-2400, Dyax Corporation) that targets MT1-MMP at its catalytic domain showed great promise in preclinical studies. DX-2400 abrogated MMP2 cleavage on tumor and endothelial cells, blocked angiogenesis, and reduced tumor formation and metastasis.[50,52] Another humanized antibody targeting the non-catalytic hemopexin domain of MT1-MMP has recently shown promise in inhibiting invasion and angiogenesis in preclinical studies.[53] The therapeutic potential of targeting MT1-MMP to inhibit invadopodia-mediated tumor invasion and metastasis holds great future promise.

ADAM proteases

The ADAMs are a family of disintegrin and metalloproteinases that are involved in a variety of biological processes, including cell

adhesion, migration, proteolysis, myoblast fusion and fertilization.[54] Here, we focus on ADAM12 because of its more established presence at invadopodia. ADAM12 has two alternatively spliced variants: ADAM12-L, which consists of pro-, metalloprotease, disintegrin, cysteine-rich, transmembrane and cytoplasmic domains, and ADAM12-S, which lacks the transmembrane and cytoplasmic domains.[55]

ADAM12 contributes to invadopodia function at multiple levels, including degrading the ECM, modulating integrin function, and functioning as a sheddase to activate growth factors.[56] ADAM12 is localized to invadopodia, binds to the scaffold protein Tks5[25] and has been found to trigger invadopodia assembly.[57] The sheddase activity of ADAM12 may contribute to the overall degradation activity of invadopodia. A recent study by Diaz *et al*.[58] demonstrated that ADAM12 expression was elevated in a Notch-dependent manner under hypoxic conditions. ADAM12 promoted the ectodomain shedding of heparin-binding epidermal growth factor (EGF)-like growth factor, which in turn induced invadopodia formation and the invasive activity of cancer cells.[58]

ADAM12 is implicated in a variety of cancers, including breast, prostate, lung, liver, brain and bone cancers, as well as aggressive fibromatosis.[59] In human breast cancer patients, Roy *et al*.[60] showed that ADAM12 is a prognostic marker, as urinary levels of ADAM12 increased along with disease stage. Transgenic mice expressing the ADAM12-S isoform driven by the MMTV-LTR promoter were bred with mice carrying the PyMT oncogene in the mammary gland; PyMT expression in the mammary gland led to rapid formation of mammary carcinomas. Tumors in mice expressing ADAM12-S developed faster than in littermates expressing PyMT alone.[61] Similarly, the ADAM12-S isoform significantly increased the ability of MCF-7 cells to migrate and invade, which led to a higher incidence of local and distant metastases *in vivo*.[62] Interestingly, cells expressing a catalytically dead mutant of ADAM12-S failed to promote tumor development, indicating that the proteolytic activity of ADAM12-S is required to promote formation of distant metastases.[62]

Serine proteases

Two transmembrane type II serine proteases of the dipeptidyl-peptidase (DPP) family, fibroblast activation protein (FAP; FAPα, also known as seprase) and DPP4, have also been associated with invadopodia. Both DPP4 and FAPα contain exopeptidase activity, and FAPα also exhibits endopeptidase activity.[63,64] Previous studies have shown that FAPα is localized at invadopodia as a complex with DPP4[65] or, alternatively, associated with α3β1 integrin, with the integrin serving as a docking site for FAPα.[66] The role of FAPα in invadopodia is currently unclear, but some studies suggest that its gelatinase activity may contribute to the overall degradation activity of invadopodia. Christiansen *et al*.[67] found that FAPα digests collagen I into smaller fragments following initial cleavage by MMP-1, suggesting that FAPα works together with other proteases to cleave partially degraded ECM components.

A key difference between FAPα and DPP4 is that expression of DPP4 is ubiquitous throughout all tissues, whereas that of FAPα is restricted to tissues undergoing wound healing and epithelial cancers,[64,68] thus making FAPα a unique factor in tumor progression. Indeed, FAPAα has been shown to be expressed in a variety of aggressive cancers, including breast, colon and ovarian cancers, and malignant melanoma.[69] In addition, genetic deletion of FAPα inhibited tumor growth in a K-ras-driven model of endogenous lung cancer and in a mouse model of colon cancer. Pharmacological inhibition of FAPα also attenuated tumor growth in these mouse models, indicating that FAPα is a promising target for therapeutic intervention.[70] In human and mouse tumors, FAPα has been shown to be expressed in stromal fibroblasts, carcinoma cells and immune cells.[69,71–73] What remains to be answered is whether and how FAPα in individual cell types contributes to tumor progress, and whether the role of FAPα at invadopodia is critical for tumor invasion and metastasis.

Previous attempts to target FAPα for therapeutic intervention have proven to be challenging. In 2003, Phase I/II clinical trials for the humanized FAPα monoclonal antibody Sibrotuzumab failed to demonstrate measurable therapeutic activity in patients with metastatic colorectal cancer,[74] with only 2 out of 17 patients having stable disease during the Phase II trial.[74] However, this antibody has not been shown to block any cellular or protease function of FAPα, which might explain the lack of therapeutic effects. In 2007, a small molecule inhibitor of FAPα, Talabostat, was developed to inhibit the protease activity of FAPα. Again, minimal clinical activity was observed in patients with metastatic colorectal cancer receiving Talabostat alone,[75] or in metastatic melanoma patients receiving Talabostat in conjunction with cisplatin treatment.[76] However, the stability of this inhibitor *in vivo* is thought be extremely poor, thus limiting its effectiveness.

Given these recent setbacks in targeting FAPα, efforts have recently turned to FAPα-mediated immunotherapy. One approach is to develop DNA vaccines to target FAPα, thus eliminating all FAPα-positive cell types in a tumor. Several groups reported that through CD8+ T-cell-mediated killing, such therapy successfully suppressed primary tumor cell growth and metastasis of implanted breast and colon tumors without obvious toxicity.[77,78] Another approach is to deliver radioisotopes specifically to the tumor site using FAPα antibodies as cargoes. Preclinical studies involving two humanized FAPα monoclonal antibodies (ESC11 and ESC14) labeled with the radiolanthanide ^{177}Lu have yielded promising results: both antibodies accumulated in human FAPα-positive xenografts and delayed tumor growth.[79] Given that FAPα is expressed in various cell types in a tumor, it is important to recognize that the effect of targeting FAPα on tumor progression cannot solely be explained by inhibition of invadopodia. However, these results suggest a unique approach to targeting components of invadopodia in human cancers.

REGULATORY COMPONENTS

As ECM is essential for cell survival and proliferation, invadopodia-mediated matrix degradation is a highly regulated process. Understanding the upstream inducing signals of invadopodia formation and function is still in its infancy. A significant number of signaling regulators, including EGFR, PDGFR, PI3 kinase (epidermal growth factor receptor, platelet-derived growth factor receptor, and phosphoinositide 3, respectively) and c-Met have been implicated in invadopodia regulation in various cancer cell lines. As many of them have critical roles in multiple cellular processes in cancer, including cell proliferation and apoptosis, it is difficult to attribute their functional impact on tumor progression specifically to invadopodia. Therefore, here we discuss a few key upstream signaling pathways that have been more uniquely implicated in invadopodia function during tumor progression and metastasis.

Phosphorylation via Src and Arg tyrosine kinases

Tyrosine phosphorylation of many core components is critical to trigger invadopodia assembly and function. Especially, two tyrosine kinases, Src and Arg, stand out as essential activators of invadopodia.

Src kinase. The Src kinase is the founding member of the Src family of non-receptor tyrosine kinases,[80] of which, Src is the only family member that is uniquely linked to invadopodia. The role of Src in invadopodia formation was first described by Chen *et al*.[81], where Rous sarcoma viral (RSV) transformation of chicken embryonic fibroblasts resulted in actin rosette or podosome

formation. In addition, RSV-mediated cellular transformation correlated with the appearance of $pp60^{Src}$ accumulation at these rosettes. Similarly, invadopodia formation was enhanced with constitutive expression of active c-Src, as evidenced by co-localization of F-actin and cortactin staining.[82] In contrast, overexpression of a kinase-inactive c-Src and knockdown of c-Src by RNAi showed decreased invadopodia formation and degradation activities.[9,83,84] Closer examination of protrusion formation in MDA-MB-231 cells revealed that overexpressing wild-type Src or constitutively active Src exhibited invadopodia extension into the collagen gel.[9]

Src kinase has been shown to have a major role in the invasive process. Expression of c-Src in SYF (src −/−, yes −/−, fyn −/−) murine embryonic fibroblasts promoted invasion, whereas Ras^{V-12} failed to do so in a Boyden chamber assay.[85] Similarly, MDA-MB-231 breast cancer cells treated with Src small interfering RNAs exhibited reduced matrix degradation and invasion through matrix-coated chambers.[84] Treatment with Src inhibitors, Dasatinib, PP2 or SU6656, in MDA-MB-231 cells reduced invasion through Matrigel, indicating the importance of Src activity for tumor cell invasion into the ECM.[86]

Although Src was originally isolated as an oncogene, recent studies suggest a more critical role for Src in tumor metastasis.[85,87,88] Specifically, c-Src activity is correlated with increased bone metastases, poor clinical prognosis and reduced survival for breast and colon carcinoma patients.[86,89,90] *Src* gene deletion in MMTV-PyMT mammary tumor models resulted in a reduction in circulating tumor cells, despite no defect in primary mammary tumor initiation and proliferation.[91] Similarly, Src small interfering RNA-treated L3.6pl pancreatic cancer cells exhibited a reduction in lung and liver metastasis.[92] c-Src was required for formation of metastatic lung colony formation by $H\text{-}Ras^{V-12}$ expressing SYF (src −/−, yes −/−, fyn −/−) murine embryonic fibroblast cells, supporting the role of Src in tumor progression.[85] Likewise, BoM-1833, a bone metastatic derivative of MDA-MB-231 cells, which were injected into recipient mice, showed increased survival and reduced bone metastases upon treatment with Src small interfering RNA.[89]

As Src activity has a prominent role in cancer progression, it is an ideal therapeutic target. The Src selective inhibitor, SU6656, has been found to inhibit Src kinase activity, as evidenced by reduced levels of phospho-Y418-Src. SU6656 was also found to significantly reduce invadopodia formation, as well as migration and invasion of the human breast cancer line MDA-MB-231.[82] KX2-391 is a first-in-class Src selective inhibitor undergoing clinical trial that targets the unique Src substrate binding site.[93] KX2-391 has shown promising preclinical data, with KX2-391 treatment in combination with paclitaxel resulting in the regression of pre-established MDA-MB-231 xenograft tumors.[94] In addition, KX2-391 treatment led to reduced metastasis formation of MDA-MB-231 tumors in the lung and liver.[94] Phase I trials in patients with solid tumors showed that KX2-391 is well tolerated and demonstrated preliminary antitumor activity, with several patients displaying halted disease progression.[95]

Abl/Arg. Similar to Src kinase, the Abl family of non-receptor tyrosine kinases, which includes c-Abl and the Abl-related gene (Arg/Abl2), has an important role in tumor progression in human leukemia, non-small cell lung cancer, breast cancer, melanoma and pancreatic cancer.[96] Specifically, c-Abl and Arg kinase activities have been shown to correlate with poorly differentiated and highly invasive breast cancer lines.[97] The role of Abl/Arg kinase was initially hypothesized in invadopodia formation due to previously known activation of Abl by Src kinases. More specifically, PDGF and EGF stimulation of fibroblast and breast cancer cells resulted in c-Abl activity through Src and Fyn phosphorylation.[97,98] Treatment of various highly invasive breast cancer cell lines with Src inhibitor SU6656 reduced c-Abl and Arg activity.[97] Src activation of Arg is required for cortactin phosphorylation and actin polymerization at invadopodia, as knockdown of Arg in MDA-MB-231 cells resulted in no cortactin phosphorylation and reduced F-actin barbed-end generation.[84]

The Abl family of non-receptor tyrosine kinases has also been localized directly to the invadopodia structure. YFP-tagged wild-type and constitutively active Arg co-localized with cortactin-positive invadopodia in Src expressing NIH3T3 cells, whereas kinase-inactive Arg expression disrupted invadopodia formation.[99] Similarly, immunofluorescence staining of Arg in MDA-MB-231 cells showed co-localization with Tks5-positive invadopodia.[84]

Abl and Arg kinases also have a significant role in tumor invasion and metastasis. Knockdown of Abl and Arg reduced the ability of MDA-MB-231, and its metastatic derivatives, to degrade ECM and invade.[97,99] Inhibition of Abl kinase activity with STI571, an Abl/Arg inhibitor, reduced invasion of MDA-MB-435S breast cancer cells in a Matrigel invasion assay.[97] MDA-MB-231 cells expressing Arg and Abl small hairpin RNA constructs showed fewer circulating tumor cells *in vivo* compared with control tumor-bearing mice.[100] Similarly, Gil-Henn *et al*.[100] showed that STI571-treated mice showed fewer circulating tumor cells compared with control mice bearing MDA-MB-231 tumors. These results indicate that Abl kinase activity is required for tumor cell intravasation.

A number of tyrosine kinase inhibitors with dual specificities toward Src and Abl family tyrosine kinases, including Dasatinib, Saracatinib, and Bosutinib, have been developed and showed promising activities against tumor invasion and metastasis in several solid tumors preclinical studies. Specifically, these inhibitors were found to significantly reduce invadopodia formation, as well as migration and invasion of the human breast cancer line MDA-MB-231.[86,101,102] In addition, treatment of mice with Dasatinib led to reduced formation of bone metastases by BoM-1833 cells.[89] Similar results have been observed in pancreatic tumors, with Dasatinib treatment leading to a reduction in primary tumor growth and metastasis formation by L3.6pl cells.[92] However, these inhibitors have shown limited activity in monotherapy trials.[93] It is important to note, however, that completed clinical trials studying the efficacy of Src/Abl inhibitors have been conducted in unselected cancer patients. Many ongoing trials using biomarkers (such as cortactin phosphorylation) to pre-select patients who are more likely to benefit from Src/Abl inhibition hold promise for the future success of Src/Able inhibitors in cancer treatment.[93]

Integrin-mediated signaling

Given that integrins are the key connection between cell protrusions and the surrounding ECM, it is not surprising that they have important roles in invadopodia regulation. Integrin clustering at invadopodia was first described in RSV transformation of chicken embryonic fibroblasts (RSVCEF). RSVCEFs cultured with fibronectin-coated beads exhibited invadopodia formation, which was associated with β1 staining.[103] Interestingly, fibronectin-positive vesicles were found to co-stain with β1 integrin; further validating the functional role of invadopodia in matrix degradation.[103] In contrast, murine embryonic fibroblasts overexpressing Src and depleted for β1 integrin exhibit reduced rosette formation.[104] In addition, treatment of SCC61 squamous cell carcinoma cells with an integrin blocking peptide or a β1 integrin blocking antibody led to a reduction in actively degrading invadopodia per cell.[105] Finally, knocking down β1 integrin in MDA-MB-231 and MTLn3 mammary adenocarcinoma cells led to a reduction in mature invadopodia formation.[106]

How integrins regulate invadopodia function has not been clearly elucidated. Various studies indicate that integrins promote invadopodia maturation by serving as a docking station for various proteases and/or activating Arg kinase for actin stabilization. Laminin peptide activation of β1 integrin in lysyl oxidase

(LOX) human melanoma cells led to increased invadopodia-mediated degradation[107] due to increased seprase/FAPAα recruitment to invadopodia by binding to β1 integrin.[7,107] More recently, β1 integrin has been found to activate Arg kinase. Fluorescence resonance energy transfer (FRET)-based experiments point to direct interaction between β1 integrin and Arg at Tks5-positive invadopodia.[106]

Changes in integrin-mediated adhesion signaling complexes are known to have an important role in tumor cell proliferation, migration and survival.[108] Specifically, loss of β1 integrin in MMTV-driven Erb2 breast tumor mice reduced Y416 c-Src phosphorylation and metastatic lesion formation in the lungs.[109] Similarly, tail vein injection of MDA-MB-435 breast cancer cells expressing a constitutively active mutant αvβ3 exhibited enhanced metastatic lung colonization.[109] To date, it is unclear which integrin subunits are the predominant forms required for invadopodia function. As the focus of current integrin-targeted therapies has been on their anti-angiogenic properties, their potential as an anti-metastatic treatment by invadopodia inhibition requires identifying and targeting invadopodia-specific integrins in the near future.

Transcriptional regulation by EMT-inducing factors

To detach from the primary tumor and invade through the surrounding tissue, carcinoma cells need to first break down cell–cell junctions, become more motile, remodel cell–matrix adhesion sites and invade through the ECM. A developmental program termed epithelial–mesenchymal transition (EMT) enables tumor cells to obtain such properties. During EMT, cells need to co-ordinate the dissociation of cell–cell adhesions and the breakthrough of basement membrane to accomplish this complex morphogenetic event.[1] Recent studies indicate a critical role of invadopodia-mediated ECM degradation during EMT.

The EMT program is orchestrated by a group of transcription factors, all of which have been implicated in tumor invasion and metastasis.[110] Specifically, the basic helix-loop-helix (bHLH) transcription factor Twist1 was shown to have critical roles in tumor metastasis in both breast tumor xenografts and in mouse skin tumor models.[111,112] Eckert *et al*.[24] found that Twist1 was required for ECM invasion by inducing the formation of invadopodia in human and mouse breast tumor cells. Twist1 was found to directly induce expression of PDGFRα, which then activates Src kinase to promote invadopodia formation.[24] Furthermore, blocking invadopodia formation by knocking down PDGFRα or Tks5 abolished the ability of Twist1 to promote tumor metastasis in mice. This study not only uncovers Twist1 as a novel upstream regulator of invadopodia formation, but also provides a direct link between invadopodia and metastasis.

Another potent inducer of EMT is transforming growth factor-β (TGFβ).[1] TGFβ promotes EMT by activation of extensive intracellular signaling that involves Smad proteins, ERK and Jagged/Notch signaling, among others.[113] Of particular interest in the context of invadopodia, Eckert *et al*.[24] showed that knocking down Twist1 blocked the ability of TGFβ to induce PDGFRα and invadopodia formation. In addition, Pignatelli *et al*.[114] also demonstrated that Hic-5, a focal adhesion adaptor protein induced by TGFβ, localized to invadopodia in TGFβ-treated MCF-10A cells. They showed that Hic-5 was phosphorylated by Src kinase upon TGFβ stimulation, and this phosphorylation was required for TGFβ-induced invadopodia formation and invasion, thereby further emphasizing the role of EMT-inducing genes in invadopodia regulation.

Matrix stiffness and mechanoregulation

Recent evidence has implicated matrix stiffness in tumor progression and increased incidence in metastasis through increased collagen deposition.[115–117] Several elegant studies show that increasing matrix stiffness without altering biochemical components of the ECM can induce a malignant phenotype, suggesting that mechanical force exerted by a stiff ECM could have a critical role in tumor invasion and metastasis.[118] In mice, LOX-mediated collagen cross-linking stiffens tumor ECM and promotes breast tumor progression.[119] Furthermore, inhibition of LOX blocks tumor invasion and eliminates metastasis formation from orthotopically grown breast tumors.[33]

Indeed, ECM rigidity is indicated in invadopodia regulation. Specifically, Alexander *et al*.[120] noted that CA1d breast cancer cells plated on increasing concentrations of gelatin showed increased invadopodia formation and ECM degradation. Interestingly, two studies showed that both CA1d and 804G bladder cancer cells exhibited increased invadopodia formation when placed on matrix substrates with increasing mechanical rigidity without changing their biochemical components.[121] Furthermore, these studies showed that invadopodia-mediated ECM degradation only increased when placed on surfaces within the kPa range, which corresponds to the stiffness in tumors.[121] Although it is not well understood how matrix rigidity regulates invadopodia formation, this study suggests that activation of p130Cas and FAK by myosin II, which acts as mechanosensors that transmit mechanical signals from ECM, could have a prominent role.[120] Given the effects of increasing matrix stiffness on invadopodia functions, it is possible that mechanical forces generated by rigid tumor matrix stiffness could modulate invadopodia function to impact tumor progression and metastasis.

THE ROLE OF INVADOPODIA IN TUMOR INVASION AND METASTASIS

The critical role of invadopodia in ECM degradation explains why the ability to form invadopodia largely correlates with the invasive and metastatic potential of tumor cells.[122] Suppressing invadopodia formation by inhibiting Src, Twist1 or Tks5 has been convincingly shown to inhibit tumor metastasis in various tumor models. Invadopodia could have critical roles during three steps of the metastatic process: invasion into the surrounding stroma, intravasation into the vasculature and extravasation (Figure 2). Direct *in vivo* visualization and assessment of invadopodia formation during the metastatic process has proven to be challenging because of the limited numbers of invadopodia-specific markers in a 3D microenvironment. Furthermore, it remains unclear whether actin-based protrusions observed in two-dimensional culture, including filopodia and invadopodia, share similar components or merge into one structure in 3D; thus confusing the issue of defining invadopodia *in vivo*.

New imaging techniques have made it possible to begin to identify invadopodia-like protrusions *in vivo* (Figure 2). Using 3D time-lapse imaging, Gligorijevic *et al*.[123] observed protrusion formation by MTLn3 rat mammary adenocarcinoma xenograft tumors growing in the mammary fat pad of SCID mice. These protrusions were positive for cortactin and proteolytic activity, as evidenced by cleaved collagen 3/4 staining, indicating the presence of invadopodia *in vivo*. Looking specifically at the intravasation, Gligorijevic *et al*.[123] used the photoconvertible Dendra2 protein to trace tumor cell intravasation *in vivo*. Control MTLn3 primary tumor cells were shown to disappear from the imaging area due to dissemination of cells into the blood stream. In contrast, knocking down N-WASP resulted in no visible dissemination. Yamaguchi *et al*.[124] observed similar invadopodia-like protrusions during tumor cell intravasation using intravital imaging. Green fluorescent protein (GFP) expressing MTLn3 cells revealed invadopodia-like protrusions extending into the blood vessel wall. These invadopodia-like protrusions were shown to help tumor cells to penetrate the ECM surrounding the blood vessel walls and to squeeze through the endothelial barrier.[124] Together, these data strongly support the notion that invadopodia are required for tumor cell intravasation (Figure 2).

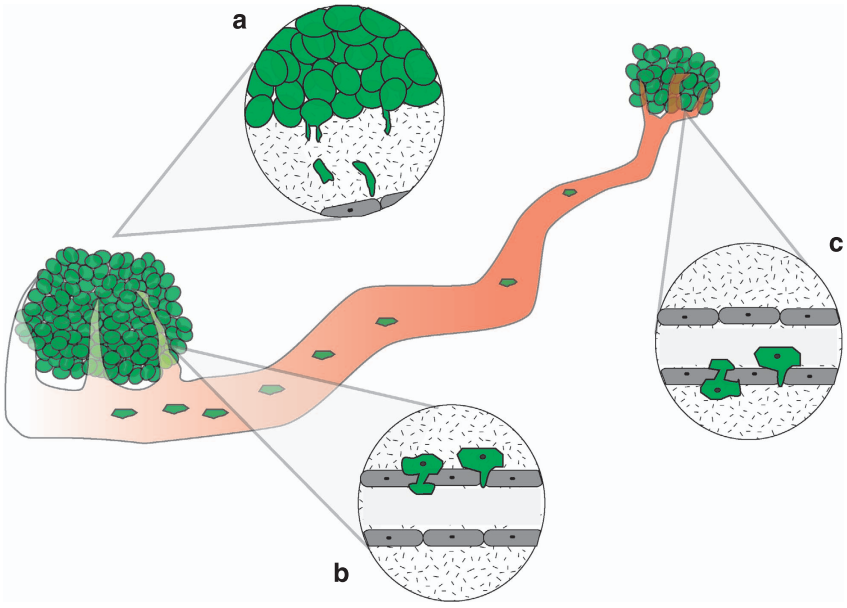

Figure 2. The role of invadopodia in metastasis progression. Tumor metastasis takes place in a series of steps: invasion into the surrounding stroma (**a**), intravasation into the vasculature (**b**), extravasation out of the vasculature (**c**) and colonization at distant sites. Given that invadopodia function to degrade ECM, invadopodia are thought to have critical roles during various steps of metastasis (**a**–**c**).

Assessment of invadopodia formation during the extravasation process is more limited; however, protrusive structures have been identified by actively extravasating tumor cells.[125,126] Specifically, time course observations of intra-meseneric vein injection of GFP-positive rat tongue carcinoma cells reveal clusters of tumor cells in the sinusoids.[125] Over time, the authors noted focal loss of basement membrane at sinusoidal areas where extravasation was taking place.[125] The loss of basement membrane indicates potential sites of active ECM degradation by invadopodia. Protrusive structures have also been identified by actively extravasating MDA-MB-435 cells expressing Twist1 upon injection into the vasculature of zebrafish.[126] Direct imaging of tumor cells in the vasculature revealed that Twist1-overexpressing cells display large, rounded protrusions,[126] suggesting that invadopodia-like protrusions by extravasating tumor cells contribute to ECM degradation and breaking through the endothelium barrier (Figure 2).

FUTURE DIRECTIONS AND THERAPEUTIC IMPLICATIONS

As discussed, recent progress suggests an essential role of invadopodia in tumor invasion and metastasis. The specific presence of invadopodia in invasive tumor cells and their unique ability to precisely co-ordinate localized ECM degradation with cell movement make them ideal targets for anti-metastasis therapies.

A number of critical issues need to be resolved to put invadopodia at the forefront of tumor metastasis research and treatment. First, although actin assembly and elongation during invadopodia initiation have been extensively studied, it remains unclear what and how matrix degrading enzymes are recruited to invadopodia to perform their functions. As broad inhibition of MMPs has not been successful in blocking metastasis, the strategy to inhibit protease recruitment could be a promising new route to specifically target ECM degradation and tumor invasion. Second, it remains unclear whether and how the molecular components and regulation of invadopodia and other actin-based membrane protrusions are different. As many invadopodia components have critical roles in various cellular processes, such as proliferation, apoptosis and migration, it is difficult to attribute all their observed activities on tumor progression to invadopodia function. Understanding such differences would further solidify the unique role of invadopodia in tumor metastasis *in vivo* and lead to more specific targeting therapies against invadopodia in tumors without affecting normal cellular functions.

To move invadopodia inhibitors into therapeutic applications against tumor metastasis, we first need to determine how to apply invadopodia inhibitors for cancer treatment. As discussed, the main function of invadopodia in tumors is to promote matrix degradation and tumor invasion, but not to regulate cell proliferation or survival. Therefore, the main utility of invadopodia inhibitors should be to prevent primary and secondary metastasis occurrences, instead of inhibiting the growth of established primary tumors and metastases. Invadopodia inhibitors could be beneficial in preventing new metastasis development in a number of metastasis-prone cancer patient groups. Using breast cancer as an example, a group of cancer patients who have already developed limited metastatic diseases, such as a single brain metastasis, may use invadopodia inhibitors to prevent secondary metastasis lesions in the brain. Also, patients that have presented lymph node positivity could benefit from invadopodia inhibitors to prevent distant metastasis development. Furthermore, recent gene expression profiling and biomarker studies make it possible to predict long-term metastasis occurrence and survival outcome in early-stage breast cancer patients. Invadopodia inhibitors, in combination with traditional chemotherapies, could potentially reduce metastasis development in a selected high-risk patient population based on such molecular profiling.

Another pressing issue that faces the entire metastasis field, including invadopodia research, is how to develop proper clinical trials to test anti-metastatic agents such as invadopodia inhibitors. As discussed above, invadopodia mainly function to promote matrix degradation, thus perturbation of their functions in tumors have little or no effect on tumor proliferation. Unfortunately, the current clinical trial system requires all anti-cancer agents to show efficacy in phase II by shrinking established primary tumors and/or distant metastases in patients before moving to phase III trials and regulatory approval. Only after approval can these agents be tested in metastasis prevention trials for more early-stage cancer patients. As invadopodia-specific inhibitors are unlikely to shrink existing tumors and metastases, these inhibitors would fail in

current phase II clinical trials even though they might be potent to prevent new metastasis occurrence. A stimulating article by Dr PS Steeg[127] has proposed that the rate of new metastasis occurrence in metastasis high-risk patients as a more appropriate end point for metastasis prevention trials. Given the unique role of invadopodia in tumor invasion and metastasis, invadopodia inhibitors need to be tested in better-designed metastasis prevention trials to explore their full potential in combating tumor metastasis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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